KINETICS OF RNA SYNTHESIS IN ROTAVIRUS INFECTED CELLS.

Moira A. Johnson.

B.Sc.(Hons) Warwick.

Thesis submitted for the degree of Doctor of Philosophy.

Department of Biological Sciences,

University of Warwick.

February 1988.

To my mother and father, Millie and Ken, for their love
and support over the past 25 years.

SUMMARY.

A method has been developed to allow the independent quantification of the level of synthesis of each strand of different viral genes during a synchronous infection of tissue culture cells. It was developed and applied to the UK tissue culture adapted bovine Rotavirus whose genome consists of 11 discrete segments of monocistronic double stranded RNA (dsRNA).

Cloned copies of the 11 genes of the UKtc Bovine Rotavirus have been sub-cloned into the polylinker region of the pGEM1 and pGEM2 transcription vectors. Using the T7 and SP6 promotors flanking this region, high specific activity RNA probes to either strand of the gene have been generated and used in Solution Hybridisations to quantitate the transcription and replication of each gene.

Quantification of the plus-strand (m-RNA) synthesis at hourly intervals throughout the growth cycle provided evidence for both quantitative and qualitative regulation of transcription. Quantitative control of transcription was demonstrated by the accumulation of much higher levels of m-RNA for some viral genes (eg:-2 and 7) than others (eg:-4 and 8). The relative molar amounts of the different viral proteins was measured at 6.5 hours post-infection and showed that they did not directly reflect the accumulation of their encoding mRNAs, providing evidence for the existence of translational control of gene expression. For example the high levels of VP6 and VP10 produced was due primarily to high translational frequency, whereas the high level of VP8 could be correlated to a high level of accumulation of its encoding m-RNA. The existence of qualitative control of viral transcription was demonstrated by the finding that the transcription of 4 genes (5,6,7 and 9) occurred independently of protein synthesis in infected cells. These data suggested that the proteins encoded by these genes may have a regulatory role in the replication cycle.

Study of the minus-strand synthesis for each viral gene indicated that the RNA replication for Rotaviruses is probably the same as that of mammalian Reoviruses.

The method described in this thesis is a generic one since with the availability of suitable c-DNA clones it could be used to achieve qualitative and quantitative analysis of viral gene expression in any viral system.

CONTENTS.

		Page
	Summary	i
	Contents	ii
	List of Figures	x
	List of Tables	xv
	Abbreviations	xvii
	Declaration	xix
	Acknowledgements	xx
	INTRODUCTION	
SECTION 1	ROTAVIRUS	1
1.i.	ROTAVIRUS AND GASTROENTERITIS.	1
1.i.a)	The importance of Rotavirus infection.	1
1.i.b)	The history of Rotaviruses.	3
1.ii.	THE ROTAVIRUS PARTICLE.	5
1.ii.a)	Morphology.	5
1.ii.b)	The Rotavirus genome.	7
	Structural analysis of the Rotavirus genome.	9
1.ii.c)	Rotavirus proteins and genome coding assignments.	11
	Proteins of UKtc Rotavirus.	12
	Proteins of other Rotavirus strains.	14
1.ii.d)	Genetics of Rotavirus.	18
	Gene reassortment.	18
	Genome rearrangements.	21
1.ii.e)	Rotavirus enzyme activities.	25
1.iii.	ROTAVIRUS REPLICATION.	26

	Virus entry.	27
	Morphogenesis.	28
	Replication of the Rotavirus genome.	29
	Proteins in transcription.	31
1.iv.	ROTAVIRUS INFECTION.	32
1.iv.a)	Rotavirus subgroups and serotypes.	32
	Subgroups.	32
	Serotypes.	33
1.iv.b)	Pathology and immunity.	35
	Pathology.	35
	Immunity.	37
1.iv.c)	Epidemiology of Rotavirus infections.	39
	Mode of transmission.	39
	Evidence for antigenic variation (Drift and Shift) in Rotavirus infections.	41
	Antigenic shift.	41
	Antigenic drift.	42
1.v.	THE ATYPICAL OR NON-GROUP A ROTAVIRUSES.	43
	The Chinese "ADRV" Rotavirus.	46
1.vi.	TOWARDS AN EFFECTIVE ANTI-ROTAVIRUS VACCINE	47
	Bovine RIT4237 Vaccine.	49
	Rhesus Rotavirus (RRV-1) vaccine.	50
SECTION 2	REPLICATION OF REOVIRUS.	54
2.a.	The family Reoviridae.	54
2.b.	Reovirus replication: An overview.	55
2.c.	The Reovirus particle.	56
2.d.	Reovirus enzyme activities.	61
2.e.	RNA of Reovirus.	62

2.f.	Activation of the transcriptional activities.	63
2.g.	Regulation of transcription.	64
2.h.	Translation of Reovirus RNA.	66
2.i.	Reovirus morphogenesis.	68
SECTION 3	MODELS OF REPLICATION IN OTHER RNA VIRUSES.	69
	Class III	70
	Class IV	70
	Class V	72
SECTION 4	RATIONALE OF THIS WORK.	74
4.a.	Background to the transcription system.	75
4.b.	SP6 polymerase.	76
4.c.	T7 polymerase.	77
4.d.	The Gemini vectors.	78
4.e.	S1 nuclease mapping.	80
	MATERIALS AND METHODS.	
	MATERIALS.	82
	METHODS.	84
1.	VIRUS GROWTH AND PURIFICATION.	84
1.i.	Growth.	84
1.ii.	Purification.	84
1.iii.	Extraction of Genomic RNA from purified virus.	85
2.	PREPARATION OF INFECTED CELL RNA.	85
3.	IDENTIFICATION OF c-DNA AND SUBCLONES.	86

3.i.	Fractionation of Rotavirus ds-RNA.	86
3.ii.	Immobilisation of the RNA.	87
3.iii.	Labelling c-DNA clones.	87
3.iv.	Hybridisation to immobilised genomic RNA.	87
4.	SUBCLONING c-DNA FRAGMENTS FROM pAT153 INTO pGEM1 AND pGEM2.	88
4.i.	Preparation of DNA.	88
4.ii.	Ligation and transformation.	89
4.iii.	Screening for recombinant colonies.	90
4.iv.	Large scale plasmid preparation.	90
5.	IN VITRO TRANSCRIPTION.	91
5.i.	Linearisation of plasmid template.	91
5.ii.	In vitro transcription.	92
	Synthesis of high specific activity transcript.	92
	Synthesis of unlabelled transcript.	93
5.iii.	Analysis of transcripts.	94
5.iii.A.	By gel electrophoresis.	94
5.iii.B.	By dot-blot.	95
6.	SOLUTION HYBRIDISATION.	95
6.i.	Preparation of standard RNA stocks.	95
6.i.A.	mRNA.	95
6.i.B.	"Unlabelled" transcript.	95
6.ii.	The Solution Hybridisation.	96
6.iii.	Analysis of dsRNA hybrids formed.	97
7.	ANALYSIS OF PROTEIN SYNTHESIS.	97
8.	PHOTOGRAPHIC RECORDS OF GELS BEARING DNA AND RNA.	98
9.	MEASUREMENT OF INCORPORATED RADIOACTIVITY	98

10. AUTORADIOGRAPHY. 99

11. PREPARATION OF dsDNA SIZE MARKERS AND LABELLING ROTAVIRUS RNA. 99

RESULTS.

CHAPTER 1

Subcloning and characterisation of Rotavirus c-DNA clones.

Introduction. 101

A. Cloning of UKtc Rotavirus RNA. 102

B. Subcloning into pGEM1 and pGEM2. 102

C. Verification of clone identity. 103

D. Size of the subcloned DNA inserts. 103

Summary. 109

CHAPTER 2

In vitro transcription using pGEM1 and pGEM2 containing Rotavirus c-DNA sequences.

Introduction. 114

A. In vitro transcription. 115

B. Characterisation of the RNA transcripts. 124

1.Orientation. 124

2.Size of dsRNA hybrids formed by the transcripts. 124

Discussion. 128

CHAPTER 3

Developement of a Solution Hybridisation assay.

Introduction. 137

A. Resuspension of RNA. 139
B. Time and temperature of hybridisation. 139
I.Time. 139
II.Temperature. 140
C. RNase digestion. 140
I.Setting up the digestion. 140
II.Temperature of RNase digestion. 142
III.Time of RNase digestion. 142
IV.Concentration of RNase A and T1. 145
V.Proteinase K treatment. 145
D. TCA precipitation. 145
E. Background CPM intrinsic to the probe RNA. 146
F. Increasing the specific activity of the probe. 148
Discussion. 148

CHAPTER 4

Quantitation of the solution hybridsation assay.
Introduction. 150
A. The calibration curve. 150
B. Analysis of infected cell RNA. 152
C. Example results and calculation. 155
D. Sensitivity of the hybridisation assay. 156
Discussion. 156
Comparison of solution hybridisation with a dot-blot based assay. 161

CHAPTER 5

Accumulation of Rotavirus plus-sense RNA during the replication cycle and

demonstration of transcriptional and translational control in gene expression.
Introduction. 165
A. Accumulation of messenger RNA. 166
1.Temporal variation. 167
2.Quantitative variation. 174
B. Polypeptide production during Rotavirus replication. 174
C. The role of translational control in Rotavirus replication. 180
Discussion. 182
CHAPTER 6 Accumulation of minus-sense RNA and the production of progeny virus particles.
Introduction. 186
A. Accumulation of minus-sense RNA. 186
B. Growth kinetics during infection. 194
C. Particle:PFU ratio of the final infected cell sample. 194
Discussion. 194
CHAPTER 7
Qualitative regulation of gene expression during virus replication.
Introduction. 199
A. Effectiveness of cycloheximide. 202
B. Selective transcription in the absence of protein synthesis. 204
Discussion. 207
GENERAL DISCUSSION. 210

APPENDIX. 220

REFERENCES. 233

ADDENDUM. 261

ADDITIONAL REFERENCES. 264

LIST OF FIGURES.

		Page
FIGURE 1.	Electron microscopy of Rotavirus particles	6
FIGURE 2.	The gene coding assignments of the UKtc and SA11 Rotaviruses.	19
FIGURE 2a.	A schematic representation of the Reovirus replication strategy.	57
FIGURE 3.	The polylinker region of pGEM1.	79
FIGURES 4-8:	Genetic origin of the Rotavirus subclones.	
FIGURE 4.	Polyacrylamide gel of Genes 1 through 9.	104
FIGURE 5.	Autoradiograph of genomic RNA hyridised to labelled c-DNA clones of Genes 1 through 4.	105
FIGURE 6.	Autoradiograph of genomic RNA hybridised to labelled c-DNA clones of Genes 5 through 9.	106
FIGURE 7.	Polyacrylamide gel of Genes 1 through 11.	107
FIGURE 8.	Autoradiograph of genomic RNA hybridised to labelled c-DNA clones of Genes 10 and 11.	108
FIGURES 9-10:	Size analysis of the c-DNA inserts subcloned into pGEM1 and pGEM2.	
FIGURE 9.		110
FIGURE 10.		111
FIGURE 11.	The molecular cloning of dsRNA of Rotavirus and insertion of c-DNA into the Gemini vectors.	118

FIGURES 12-15: Agarose gel electrophoresis of high specific activity transcript RNA.

FIGURE 12. Analysis of RNA transcripts for Genes 5 and 6. 120

FIGURE 13. Analysis of RNA transcripts for Genes 7 and 8. 121

FIGURE 14. Analysis of RNA transcripts for Gene 9. 122

FIGURE 15. Analysis of RNA transcripts for Gene 10. 123

FIGURE 16. Determination of transcript orientation by Dot-blot analysis. 125

FIGURES 17-18: Summary of the "sense" of the RNA transcripts.

FIGURE 17. 126

FIGURE 18. 126

FIGURES 19-21: Polyacrylamide gel analysis of dsRNA hybrids formed between transcript and Rotavirus genomic (ds) and m-RNA.

FIGURE 19. Analysis of the hybrids formed between Gene 3 transcripts and Rotavirus RNA. 130

FIGURE 20. Analysis of the hybrids formed between Gene 6 transcripts, Rotavirus RNA and infected cell RNA. 131

FIGURE 21. Analysis of the hybrids formed between Gene 11 transcripts and Rotavirus RNA. 132

FIGURE 22. The structure of the T7 termination signal and potential hairpin-loop structures in the Gene 10 (400bp) subclone. 135

FIGURE 23. Calibration curve for high specific activity transcript. 151

FIGURE 24. Messenger RNA detection at 8.5 hours post infection using a Gene 8 probe. 153

FIGURE 25. Minus-sense RNA detection at 8.5 hours post infection using a Gene 8 probe. 154

FIGURE 26. Example results: Gene 11 plus-sense RNA detection (mRNA). 157

FIGURE 27. Conversion of RNase resistant radioactivity to molecules per cell for Gene 11 plus-sense RNA. 159

FIGURES 28-29: Dot-blot hybridisation with the high specific activity transcript used in Solution Hybridisation.

FIGURE 28. Hybridisation to a minus-sense Gene 7 high specific-activity transcript (Clone 17 transcribed with T7 polymerase). 162

FIGURE 29. Hybridisation to a plus-sense Gene 7 high specific activity transcript (Clone 17 transcribed with SP6 polymerase). 162

FIGURE 30 PARTS A-K.
Accumulation of Rotavirus messenger RNA during a simultaneous infection.

FIGURE 30 A. Accumulation of Gene 1 plus-sense (messenger) RNA. 168

FIGURE 30 B. Accumulation of Gene 2 plus-sense (messenger) RNA. 168

FIGURE 30 C. Accumulation of Gene 3 plus-sense (messenger) RNA. 169

FIGURE 30 D. Accumulation of Gene 4 plus-sense (messenger) RNA. 169

FIGURE 30 E. Accumulation of Gene 5 plus-sense (messenger) RNA. 170
FIGURE 30 F. Accumulation of Gene 6 plus-sense (messenger) RNA. 170
FIGURE 30 G. Accumulation of Gene 7 plus-sense (messenger) RNA. 171
FIGURE 30 H. Accumulation of Gene 8 plus-sense (messenger) RNA. 171
FIGURE 30 I. Accumulation of Gene 9 plus-sense (messenger) RNA. 172
FIGURE 30 J. Accumulation of Gene 10 plus-sense (messenger) RNA. 172
FIGURE 30 K. Accumulation of Gene 11 plus-sense (messenger) RNA. 173
FIGURE 31. Rotavirus specific polypeptides fractionated by P.A.G.E. 176
FIGURE 32 A. Accumulation of Gene 1 minus-sense (complementary) RNA. 187
FIGURE 32 B. Accumulation of Gene 2 minus-sense (complementary) RNA. 187
FIGURE 32 C. Accumulation of Gene 3 minus-sense (complementary) RNA. 188
FIGURE 32 D. Accumulation of Gene 4 minus-sense (complementary) RNA. 188
FIGURE 32 E. Accumulation of Gene 5 minus-sense (complementary) RNA. 189
FIGURE 32 F. Accumulation of Gene 6 minus-sense (complementary) RNA. 189

FIGURE 32 G. Accumulation of Gene 7 minus-sense (complementary) RNA. 190

FIGURE 32 H. Accumulation of Gene 8 minus-sense (complementary) RNA. 190

FIGURE 32 I. Accumulation of Gene 9 minus-sense (complementary) RNA. 191

FIGURE 32 J. Accumulation of Gene 10 minus-sense (complementary) RNA. 191

FIGURE 32 K. Accumulation of Gene 11 minus-sense (complementary) RNA. 192

FIGURE 33. One-step growth of Rotavirus: Production of infectious progeny virions. 195

FIGURE 34. Selective transcription in the absense of protein synthesis. 205

FIGURE 35. Summary of the Solution Hybridisation assay for the accumulation of specific strands of individual RNA species during a Rotavirus infection.

LIST OF TABLES.

		Page.
TABLE 1.	Summary of available information on Reovirus proteins.	58
TABLE 2.	Transcription and translation frequencies of the 10 Reovirus Genes.	67
TABLE 3.	Characteristics of Rotavirus subclones in pGEM1 and pGEM2.	112
TABLE 4.	Characteristics of the RNA transcripts used in hybridisation assays.	133
TABLE 5.	Optimum temperature for RNA-RNA hybrid formation.	141
TABLE 6.	Optimum temperature for RNase digestion following hybridisation.	143
TABLE 7.	Variation of the duration of hybridisation and RNase digestion.	144
TABLE 8.	RNase resistance of "Medium" and "High" specific activity transcripts.	147
TABLE 9.	Conversion of RNase resistant radioactivity to molecules of RNA per cell for gene 11 plus-sense RNA.	160
TABLE 10.	Methionine content of the Rotavirus proteins.	177
TABLE 11.	Rotavirus protein production at 6.5 hours post-infection (relative to VP11).	179
TABLE 12.	Translation and transcription of the 11 Rotavirus gene products.	181

TABLE 13. Inhibition of protein synthesis by cycloheximide. 203

TABLE 14. mRNA accumulation in the presence of cycloheximide. 206

APPENDIX

TABLE 15. The accumulation of Plus-sense RNA. 221

TABLE 16. The accumulation of minus-sense RNA. 227

TABLE 17. A summary of the Rotavirus proteins. 232a

ABBREVIATIONS.

βME	Beta Mercaptoethanol
BSC-1	African Green Monkey Kidney (epithelial) cells
BSA	Bovine Serum Albumin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleoside triphosphate
DS	Double-shell
ds	Double-strand
E.coli	Escherichia coli
EDIM	Epizootic Diarrhoea of Infant Mice
EDTA	Diaminoethanetetra-acetic acid, disodium salt
EGTA	Ethyleneglycol-bis-(aminoethyl ether) N,N' tetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EM	Electron Microscopy
g	Gravitational force
GMEM	Glasgow minimal essential medium
IAHA	Immune Adherence Haemagglutination Assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISVP	Intermediate sub-viral particle
MA104	African Green Monkey Kidney (epithelial) cells
MOI	Multiplicity of Infection
MOPS	3-[N-Morpholino] propanesulfonic acid
M.wt.	Molecular weight
NCDV	Nebraska Calf Diarrhoea Virus
NTP	Nucleoside triphosphate

OD	Optical Density
OSU	Ohio State University Tissue Culture Adapted Porcine Rotavirus.
PAGE	Polyacrylamide Gel Electrophoresis
PFU	Plaque Forming Unit
PI	Post-infection
PIPES	1,4-Piperazinediethanesulfonic acid
PPO	2,5-Diphenyloxazole
rATP	Ribo-adenosine triphosphate
rCTP	Ribo-cytidine triphosphate
rGTP	Ribo-guanosine triphosphate
RNA	Ribonucleic acid
RNase	Ribonuclease
rUTP	Ribo-uridine triphosphate
SA11	Simian 11 Rotavirus
SDS	Sodium Dodecyl Sulphate
Sp	Species of Rotavirus genomic RNA (gene)
SS	Single-Shell
ss	Single-strand
SVP	Sub-Viral particle
TCA	Tricholoroacetic acid
UKtc	United Kingdom Tissue Culture adapted Bovine Rotavirus
UN	Uninfected
VP	Virus specific polypeptide

DECLARATION.

I hereby declare that the work described in this thesis was conducted by myself under the supervision of Dr M.A. McCrae. All sources of information have been specifically acknowledged by means of reference.

None of the work contained in this thesis has been used in any previous application for a degree.

M. Johnson.

Moira A. Johnson.

ACKNOWLEDGEMENTS.

I should like to thank Dr McCrae for all the advice, help, critism and moral support given over the last three years, and Prof. Roger Whittenbury for having me in the department since 1981.

Thanks are also due to members of the Rotavirus group past and present, especially Janet McCorquodale, Olwynne Tarlow, Gill Scott and Roger Jenkins whom I also thank for proof-reading the manuscript.

The help and advice given by Dr John Shuttleworth during the early stages of this work is gratefully acknowledged.

Finally a special word of thanks to those people who have cared so much; my parents, Ruth, Malcolm, and Peter and Joan.

This work was supported by the Medical Research Council.

INTRODUCTION.

INTRODUCTION

ROTAVIRUS

1.1. ROTAVIRUS AND VIRAL GASTROENTERITIS

a) THE IMPORTANCE OF ROTAVIRUS INFECTION

Rotaviruses are now recognised as the single most important cause of acute viral gastroenteritis in the young of humans and all the major species of domestic livestock (Flewett et al 1974). In the developing countries in Asia, Africa and Latin America Rotavirus infections cause significant infant mortality, being responsible for approximately 6% of all diarrhoeal episodes, and 20% of all diarrhoeal deaths in children under 5 years of age (DeZoysa and Feacham 1986), accounting for between 5 and 10 million infant deaths per annum (Kapikian et al 1986). Rotavirus infections are also a pathogen of major importance in the developed world, where the problems they pose can be divided into two categories. Firstly they cause huge economic losses in the agricultural industry; wasting and eventual death of livestock is estimated to cost millions of pounds in the British Dairy industry alone. Secondly, Rotavirus infections are responsible for a large proportion of the infant morbidity in developed countries. The cost of treating Rotavirus infections is high; each year in Melbourne Australia, 300 children aged less than 24 months are admitted to and treated in hospital, a trend which is reflected in developed nations throughout the world (Bishop

1984); It is estimated that Rotavirus infections account for between 50 and 60% of all cases of acute infantile diarrhoea admitted to hospital (Bishop 1984).

In an attempt to try to reduce the number of infant deaths caused by diarrhoea in the developing countries, in 1978 the World Health Organisation launched a programme to encourage the use of a simple but effective method of diarrhoeal control, known as Oral Re-hydration Therapy. During Rotavirus infection, the columnar epithelial cells lining the small intestine are shed, leaving behind only immature cells which have an underdeveloped glucose-coupled sodium transport mechanism (Davidson et al 1977 and section 1 iv part b). The small intestine is then unable to take up the sugar present in milk, which remains in the lumen of the intestine where it exerts an osmotic effect causing the retention (and therefore non-absorption) of water leading to the rapid and severe dehydration which is ultimately the cause of death. The co-administration of sodium and glucose is an effective treatment for the dehydration, as they are absorbed together thus allowing the retention of water by the body (Agarwal 1979, Santosham et al 1982). The main problem facing the widespread use of this method is one of education and communication, it remains difficult to ensure that even such a simple treatment is administered properly.

In recent years there has been a great deal of interest in the development of an effective anti-Rotavirus vaccine (Section 1(vi)). In 1985 the World Health Organisation estimated that the use of Rotavirus immunisation could potentially reduce overall diarrhoeal morbidity rates by 2-3%, and mortality rates by 6-10% in children under 5 years of age in the developing countries

(DeZoysa and Feachem 1986). Such an advance would also be welcomed by the developing countries, mainly for use in the agricultural industry.

b) THE HISTORY OF ROTAVIRUSES

In 1947, Cheever and Mueller reported the first definite case of acute viral gastroenteritis in a laboratory colony of suckling mice. Attempts to isolate a bacterial agent suggested but could not conclusively prove that the cause could be a salmonella infection. In 1963, the aetiologic agent of this disease which is now known as Epizootic Diarrhoea of Infant Mice (EDIM) was formally identified as a virus (Adams and Kraft 1963). electron micrographs, probably the earliest pictures of a Rotavirus to be published showed 65 and 75 nm particles in mouse jejunum. These two particles which we now know to be single and double-shelled particles (Section 1(ii)a.) were tentatively identified as the EDIM virus. Since this first identification, Rotaviruses have been isolated from many sources (for a review see McNulty 1978). When first visualised they were termed Orbivirus-like or Reovirus-like as morphologically they resembled viruses of these already defined groups (Leece et al 1978). However it soon became apparent that this new group of viruses was distinct from either of these groups and the name "Rotavirus" was proposed (Flewett et al 1973,1974). It is now known that Rotavirus infections occur in many mammalian and avian species including cattle (Mebus et al 1969, 1971a,1971b, Woode et al 1974), sheep (Snodgrass et al 1978), pigs (Leece et al 1978) and turkeys and chickens (McNulty et al 1981).

In 1974 and 1975 the first reports of human Rotavirus infections were documented (Bishop et al 1974, Cruickshank et al 1974, Kapikian et al 1974, Davidson et al 1975, Shepherd et al 1975). Serological studies showed that the Reovirus-like particles were antigenically related to the EDIM virus and to Nebraska Calf Diarrhoea virus (Davidson et al 1975), and in 1976 Woode et al reported that the viruses isolated from acute gastroenteritis of children, calves, piglets, mice and foals were morphologically indistinguishable from each other and from the Simian SA11 virus and the "O" agent which were already being cultivated in tissue-culture (Malherbe and Strickland-Chomley 1967). However they also reported that these viruses differed from each other in their antigenic properties and in their ability to cross-neutralise and so it became apparent that the group "Rotavirus" consists of several similar, but distinct viruses causing one disease in many different species.

Biochemical studies revealed that the Rotavirus genome consisted of double-stranded RNA (Rodger et al 1975, Newman et al 1975) divided into discrete segments (Todd and McNulty 1976) and as early as 1977 it became apparent that there were genomic as well as antigenic differences between Rotaviruses of different origin (Todd and McNulty 1977). Since this time many advances have been made in the study of Rotaviruses, and as will become clear knowledge and understanding of this virus group has advanced from their recognition as an important pathogen to their molecular characterisation and the developement of candidate vaccines.

1H. THE ROTAVIRUS PARTICLE

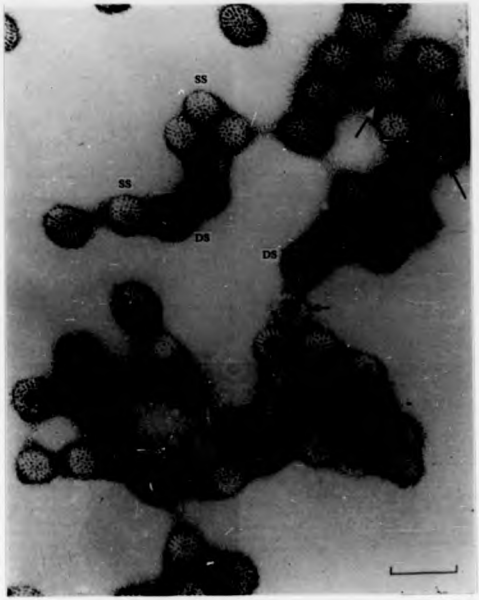
a) MORPHOLOGY

It was the distinctive morphology of the Rotavirus particles which gave this group its name (Flewett et al 1973,1974). When studied by negative staining contrast electron microscopy the particles are seen to consist of an icosahedral core of approximately 38nm diameter, surrounded by an inner layer of capsomers which project outwards from the core. Attached to these projections are the outer-layer capsomers, which form the well defined margin of the complete virus particles which are approximately 68nm in diameter (Figure 1). These three features together form the characteristic wheel-like appearance of the "Rotavirus" particles, derived from the latin word "Rota" meaning "wheel" (Flewett et al 1973,1974). The outer shell of the Rotavirus particle may be removed either physically or enzymatically to yield single-shelled particles, which are about 10nm smaller than the intact particles. Both field isolates, and tissue culture grown stocks of Rotavirus often contain both double and single-shelled particles (Bridger and Woode 1976) as shown in Figure 1.

The arrangement of the capsomers in the two layers of the virus particle has not yet been fully resolved. Defining the structure of the icosahedron has been difficult because some of the capsomers are formed by the sharing of sub-units. Based on the structure of other icosahedral viruses, structures for the double-shelled particle have been proposed (Esparza and Gil 1978) however its exact nature remains to be defined. Because of this

FIGURE 1: ELECTRON MICROSCOPY OF ROTAVIRUS PARTICLES

Human Rotavirus was prepared by glycerol-potassium tartrate density-gradient centrifugation and stained with 0.5% Uranyl Acetate. Several types of particle are apparent: "DS" denotes double-shelled particles, "SS" single-shelled particles and an example of a degraded particle is indicated by the short arrow. Long arrows indicate large ring-shaped morphological units seen on the surface of some particles. The bar marker represents 100nm. Reproduced from Palmer and Martin (1982).


SS
SS
DS
DS

unique morphology and the fact that an infected animal may excrete up to 1 X 10^{11} virus particles per gram of faeces, Rotavirus infection can be rapidly diagnosed by electron microscopy.

From their enteric nature one would expect Rotaviruses to be relatively stable entities. The morphology of Rotavirus remains unaltered by non-ionic detergents, heat, centrifugal force, high salt concentrations and acidic pH down to pH 3.5 (Palmer et al 1977). However, infectivity of SA11 is labile to alkaline conditions exceeding pH10 while treatment with low concentrations of the chelating agents EDTA and EGTA and with various salt solutions has suggested that calcium ions may play a role in maintaining the integrity of the outer capsid shell. Indeed Cohen et al (1979) observed that the outer capsid shell glycoproteins are removed by calcium chelation, and that the resulting single-shelled particles are non-infectious. This is consistent with the observation that only double-shelled particles are infectious (Elias 1977).

The infectivity of a virus stock is enhanced by the proteolytic enzymes pancreatin, trypsin or elastase (Babiuk et al 1977, Theil et al 1978). This property has been attributed to an effect on the outer capsid protein VP3 (in SA11, equivalent to VP4 of the UKtc strain) and has been used to assist the adaptation of field isolates to growth in tissue culture (Espejo et al 1981, Estes et al 1981) (see section 1iic).

b) THE ROTAVIRUS GENOME

Polyacrylamide gel analysis of numerous Rotavirus strains has shown that all Rotaviruses contain 11 segments of RNA present in equimolar amounts within the virion (Newman et al 1975, Rodger et al 1975). The 11 segments fall into 4 size classes, forming characteristic and diagnostic genome profiles of the Rotaviruses. Many estimates of the sizes of the genome segments have been made, but the most reliable is that of Rixon et al (1984) who measured the length of the UKtc Rotavirus genes by electron microscopy. They reported that the genome segments range in molecular weight from 2.25 X 10^6 (3409 bp) to 0.42 X 10^6 (637 bp), giving a total genomic molecular weight of 12 x 10^6 (Holmes 1983). Whether the dsRNA exists as these discrete segments within the virion, or is linked in some way is presently unknown. Genetic studies indicate that in functional terms the viral genome is truly segmented since independent reassortment of Rotavirus genes during mixed infections occurs indicating that at least during part of the replication cycle the genes exist as unlinked independent units (section 1(ii)d).

Polyacrylamide gel analysis of the genome from Rotavirus isolates has revealed that they can be distinguished on the basis of the migration pattern of their RNA (Kalica et al 1976, Todd and McNulty 1977, Verly and Cohen 1977), and has also been used as a diagnostic tool (Herring et al 1982). More recently it has been demonstrated that a number of different electropherotypes may be present simultaneously within a population (section 1iid) which may have important implications in the epidemiology of infections, although it should be emphasised

that a genomic variation does not necessarily reflect an antigenic variation and so it is not possible to predict the serotypic or pathologic characteristics of a new isolate simply by looking at its genome profile.

From the analysis of RNA electropherotypes it has become apparent that among human Rotaviruses there are two main patterns of RNA migration, termed the "long" and the "short" electropherotypes. This is determined by a change in migration of genome segment 11; in the "long" pattern the genome segments migrate in strict numerical order, with Segment 1 migrating the slowest and Segment 11 migrating the fastest, while in the "short" pattern genome segment 11 migrates between segments 9 and 10. The Sp11 of the "short" profile corresponds to the Sp10 of the "long" profile as was demonstrated using gene coding assignments (Dyall-Smith and Holmes 1981). It has been found that there is a correlation between these "long" and "short" electropherotypes and the subgroup antigen detected by the ELISA-IAHA tests (Kalica et al 1981b, Rodger et al 1981). However it appears that this correlation is coincidental since the antigen which defines the subgroup is encoded by genome segment 6 (Garbarg-Chenon et al 1986). As more strains of Rotavirus are examined the significance (if any) of this observation may become apparent. All of the animal Rotaviruses analysed to date, with the exception of the EDIM virus, have long electropherotypes and belong to subgroup 1 (See addendum 1).

STRUCTURAL ANALYSIS OF THE ROTAVIRUS GENOME

The terminal structure of the Rotavirus genomic and messenger RNAs has been studied using the techniques of S1 nuclease digestion and direct sequencing (McCrae and McCorquodale 1983a,b) and T1 RNAase fingerprinting (Clarke and McCrae 1981,1983). It has been shown that at the 3' terminus of each RNA segment there is an oligonucleotide which is completely conserved between all RNA species from all the virus isolates examined. For the plus-sense (coding) strand this is an octanucleotide (5'-AUGUGACC-3'). The sequence in the minus (complementary) strand is of approximately the same length but is non-complementary to that on the plus-strand, therefore the genomic RNA segments are dissimilar at their 5' and 3' terminal sequence. Immediately internal to this region of absolute conservation, a region of approximately 40 nucleotides has been defined (Clarke and McCrae 1983) whose T1 RNA fingerprint is fully conserved for a particular genome segment. These RNA segment specific terminal fingerprints were found to be conserved between Rotavirus RNAs isolated from different animal species and also those from the same animal species yet showing gross divergence of internal RNA sequence in a particular gene. These observations indicate that the terminal sequences of the genomic RNA segments have been selectively conserved during evolution and therefore suggest that they are important to the Rotavirus replication cycle. It is possible that these conserved regions may play a regulatory role in replication; possibilities include modulation of RNA transcription or replication rates, control of the level of transcription of the RNA species, and regulation of genome segment selction during viral assembly (McCrae and McCorquodale 1983b). Further, it has been demonstrated that the mRNA

transcribed from genomic RNA in-vitro is a full-length copy, there is no truncation of transcripts (McCrae and McCorquodale 1983b). Therefore, if there is a structural basis for overcoming the mRNA's dichotomy of function (mRNA functions both as template for protein synthesis and for the synthesis of the complementary strands of the genome (Section 1(iii)b) it is very different from that used by influenza virus (Section 3).

The description of the Rotavirus genome presented in this section refers exclusively to the "group A" or "Typical" Rotaviruses, virus isolates not in Group A are considered in a separate section (Section (1v)).

c) ROTAVIRUS PROTEINS AND GENOME CODING ASSIGNMENTS

Rotavirus specific polypeptides in virions and virus infected cells have been studied by several groups of workers. The earliest reports came from Newman et al (1975) and Rodger et al (1975), and were rapidly followed by large numbers of other publications (Bridger and Woode 1976, Obijeski et al 1977, Rodger et al 1977, Todd and McNulty 1977, Cohen et al 1979, Espejo et al 1980a, 1981, Estes et al 1981, McCrae and Faulkner-Valle 1981). However, probably because the methodology used in these cases differed in detail from each other, the data obtained often showed large discrepancies, and will therefore not be reviewed here. Recent attempts have been made to unify this data particularly with respect to the SA11 Rotavirus, the most studied of the cultivable Rotavirus strains.

Please see Table 17 in Appendix for a summary of the Rotavirus proteins.

PROTEINS OF UKtc ROTAVIRUS

The synthesis of the UKtc Rotavirus polypeptides has been studied by pulse-chase analysis of infected cells followed by PAGE (McCrae and Faulkner-Valle 1981). This showed that there are at least 16 virus specific polypeptides ranging in molecular weight from 20K to approx 125K, and that infection with virus rapidly causes the cessation of host-cell protein synthesis (within four hours post-infection in a single-step growth at 37℃ where maximum virus yield occurs at 12 hours post-infection). The polypeptides were labelled according to the scheme of Mason et al (1980), in which proteins are numbered according to decreasing molecular weight preceeded by the letters "VP".

Comparison of the protein profile obtained from infected cells with the protein composition of labelled single and double shelled Rotavirus particles allowed deduction of the location of the polypeptides. In the UKtc system 4 outer-shell polypeptides and 6 inner shell polypeptides were tentatively identified at this time (McCrae and Faulkner-Valle 1981). Since that time provisional functional assignments have been made for a number of these proteins.

VIRUS SPECIFIC NON-STRUCTURAL POLYPEPTIDES

The proteins designated VP8 and VP10 are the only proteins clearly not present in the virus particle, and are therefore termed non-structural. To date nothing is known about the nature or function of VP8. The gene encoding VP10 has been cloned and sequenced from SA11 (Both et al 1983) UKtc (Baybutt and

McCrae 1984) and Human Wa (Okada et al 1984), but as yet nothing is known about its function in virus infected cells. In pulse-chase studies using [3-H]-Glucosamine and the glycosylation inhibitor Tunicamycin it has been shown that VP12 is the nonglycosylated precursor of VP10, which is then modified further (this is thought to be a second glycosylation step) to yield VP10c which appears to be the final product. Apparently not all of the VP12 is available for these post-translational modifications since even after a chase of upto 2 hours some VP12 remains in the infected cell profile (McCrae and Faulkner-Valle 1981).

VIRUS SPECIFIC STRUCTURAL POLYPEPTIDES

Of the 9 remaining polypeptides it is now known that 7 are definitely structural components of the Rotavirion. Of these, VP1, VP2, and VP3, are located in the inner shell of the virion. The proteins responsible for catalysing and controlling transcription, replication and morphogenesis have not yet been identified. Because of the close proximity of these three inner shell proteins with the viral RNA it is postulated that they may interact with the genome in either catalytic or regulatory roles during the stages of replication mentioned above. However as yet there is no evidence to support these suggestions. VP6 also present on the inner capsid layer is known to be the subgroup antigen (Section 1(iv)a). This antigen appears to be common to Rotaviruses from different animal species which are serologically distinct and it would therefore appear that VP6 is evolutionarily quite stable. Recently VP6 has been expressed to high levels in the Baculovirus expression system (Estes et al 1987), which will

facilitate analysis of its role in replication and morphogenesis of the virus. (See addendum 1 for a more detailed description of VP6). A fourth protein VP5 has not definitely been assigned to either the inner or the outer shell.

The outer capsid shell of UKtc Rotavirus is composed mainly of VP4 and VP7c. VP7 was found to be a glycosylated protein, first being synthesised as VPr7 (Tunicamycin studies; McCrae and Faulkner-Valle 1981). VPr7 is glycosylated to form VP7 which is then further glycosylated to yield VP7c, which is now known to be the major neutralisation antigen of UKtc Rotavirus and the antigen which defines serotype. VP4, also on the outer surface of the virus (from studies with other strains) is now known to play a part in neutralisation of infectivity (Part B this Section).

The remaining two proteins VP8 and VP11 have not yet been unequivocably designated non-structural or structural. It is thought that VP8 is probably non-structural, and interestingly this protein has been shown to have RNA binding properties in vitro which suggests that it may play a part in viral morphogenesis, or regulation of transcription (McCrae and Baybutt, personal communication).

Although the functions of several of the structural proteins of UKtc Rotavirus have been determined, the identity of those proteins involved in RNA synthesis and regulation of the different stages in the replication cycle remain unknown.

PROTEINS OF OTHER ROTAVIRUS STRAINS

Most of the detailed structural analysis of the Rotavirus proteins has concentrated on the SA11 strain, and it is probable that this work will be directly applicable to the UKtc Bovine strain. For this reason a summary of the available information relating to the two most studied SA11 proteins, VP3 and VP7 is included, which together with data from other strains such as the Bovine Rotavirus (BRV), gives a more complete picture of the viral proteins and their functions.

VP3 (SA11, HUMAN Wa) OR VP4 (UKTC)

It was shown by Espejo et al (1981) and then by Estes et al (1981) that the primary SA11 gene 4 product, VP3 (88K) undergues proteolytic cleavage to produce proteins termed VP5* (60K) and VP8* (28K), this event has not yet been formally identified in UKtc Rotavirus (McCrae and Faulkner-Valle 1981). It was further shown by Offit et al (1983) that this cleavage step is responsible for the protease activation of infectivity of Simian SA11, Bovine NCDV and Human Wa Rotavirus strains, and the cleavage sites themselves are conserved among several human Rotavirus strains examined (Lopez et al 1986). Further functions assigned to this protein complex are that it is a second neutralisation antigen, segregating independently of the first (VP7) (Hoshino et al 1985, Offit and Blavat 1986) it is the haemagglutinin molecule, is responsible for protease enhanced plaque formation (Kalica et al 1983) and is also involved in the restriction of growth of fastidious Rotavirus in tissue culture (Greenberg 1983a). VP3 also determines the virulence of SA11 and NCDV reassortants in a mouse experimental model (Offit and Blavat

1986, Offit et al 1986a,b) and it has been shown that virulence of SA11 strains may be manipulated by modification of gene segment 4 (Offit et al 1986a). The importance of gene segment 4 in the determination of virulence has also been illustrated by Gorziglia et al (1986), who sequenced VP8 (the N-terminal portion of VP3) the cleavage region, and the amino terminus of VP5 (see beginning of this section) of Rotavirus strains isolated from symptomatic and asymptomatic infections of human neonates. The isolates sequenced represented all four human Rotavirus serotypes and it was found that 48 amino acid residues in the region sequenced were conserved; in asymptomatic isolates one amino acid was conserved at each of these positions while in symptomatic isolates a different amino acid was conserved. This suggests that VP3 (the fourth gene product) of virulent and avirulent human strains represent two lines of divergent evolution from a common ancestor and it is therefore possible that this sequence dimorphism may be responsible at least in part for the difference in virulence between the two groups (Gorziglia et al 1986).

VP7c

VP7c is the major Rotavirus neutralisation antigen. It has recently been shown by Chan et al (1986) that the gene encoding SA11 VP7 (gene 9) is infact bicistronic, containing two in-phase initiation codons. Translation from the first AUG produces a glycoprotein containing a cleaved signal sequence (37K) while translation from the second AUG also gives rise to a glycoprotein but with an uncleaved signal sequence (35.3K). Both products are immunoprecipitated by anti-VP7 antisera, both are

expressed at similar times post-infection and both were found in Rotavirus particles from several different strains. This heterogeneity apparently reflects mutations in the glycoprotein gene but whether it has any biological significance remains unknown.

One final point worthy of mention is the report that two of the Bovine Rotavirus proteins exhibit RNA binding properties. Boyle and Holmes (1986) observed that two of the Bovine Rotavirus (BRV) proteins bind specifically to RNA in RNA-overlay protein blot assays. VP2 bound single stranded RNA in preference to dsRNA, while a protein designated NS31 (a 31K non-structural protein) bound ss and dsRNA equally well. This observation suggests that VP2 and NS31 are possibly involved in interaction with RNA during transcription, replication or assembly of the virus particles. VP2 is equivalent to the VP2 of the UKtc strain and is on the inner capsid shell. NS31 could be equivalent to VP8 or VP9 in the UKtc strain on the basis of molecular weight, and as previously stated VP8 is thought to have a strong affinity for RNA.

From the study of UKtc Bovine Rotavirus (McCrae and Faulkner-Valle 1981) it appeared that the number of primary gene products was 11, the same as the number of genomic RNA segments. It was therefore postulated that the 11 dsRNA molecules would be monocistronic, as had already been established for the 10 dsRNA segments of Reovirus Type 3 (McCrae and Joklik 1978), although the S1 gene is now known to be bicistronic (Ernst and Shatkin 1985, Jacobs and Samuel 1985). By in-vitro translation of genomic RNA (McCrae and McCorquodale 1982a) it was shown that the Rotavirus genes encode the virus-specific proteins having the same numerical

notation, with the exception of Sp7 which encodes VP8 and Sp8 which encodes VP7. Similar studies have been carried out with SA11 Rotavirus (Sp1-6; Smith et al 1980, Sp10 and 11; Dyall-Smith and Holmes 1981, Sp7-9 ; Kantharidis et al 1983) and both sets of data confirm that for Rotavirus one gene encodes one primary polypeptide product with the exception of SA11 gene 9 which has recently been shown to be bi-cistronic (Chan et al 1986). The gene coding assignments and protein products for the SA11 and UKtc strains of Rotavirus are summarised in Figure 2.

d) GENETICS OF ROTAVIRUS

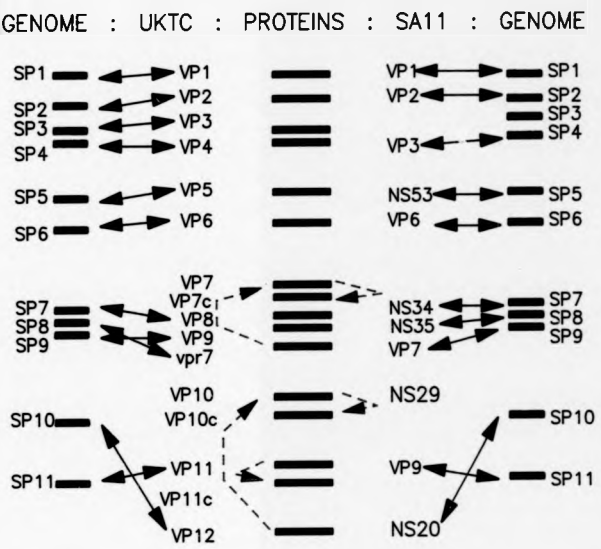
GENE REASSORTMENT

Like other segmented genome viruses, such as Reovirus and Influenza virus, Rotavirus can undergo high frequency random reassortment during mixed infection in tissue culture cells (Matsuno et al 1980) indicating that at least at the time when reassortment occurs, the segments are not joined together but exist as independent units. Coinfection of tissue culture cells with two electrophoretically and biologically distinct Rotavirus strains generates progeny bearing different combinations of the parental genes. By examining the coincidence between a given biological characteristic and the derivation of the genes it has been possible to assign several functions to specific genes: Gene 4 has been shown to encode VP4 of the UKtc strain (McCrae and McCorquodale 1982) and VP3 of the SA11 strain (Smith et al 1980), a protein with many functions already described (see section 1(ii)c). By using genetic reassortment methods, Gene 4

FIGURE 2: THE GENE CODING ASSIGNMENTS OF THE UKTC AND SA11 ROTAVIRUSES.

The gene coding assignments of the 11 ds RNA segments of the UKtc (McCrae and McCorquodale 1982) and SA11 (Estes et al 1983, Mason et al 1983) Rotavirus strains are summarised diagrammatically. The centre column of Figure 2 shows the protein profile of the UKtc strain and to its left the nomenclature of the primary translation and modified products. Equivalent proteins identified in SA11 are indicted to the right of this profile although it should be noted that they do not necessarily migrate to the same positions as the UKtc proteins. Since there is no standardisation of the nomenclature used for the SA11 proteins the system used by Estes et al is applied to this Figure. To date this group of workers has failed to assign a name to the product of gene 3, although such a product has been identified by others (Smith et al 1980).

Please refer to Table 17 (Appendix): Since submission of this thesis, Liu et al (1988) have identified the protein product of Genome segment 3, and propose that the SA11 protein VP3 (the product of Gene 4) be known as VP4 from now on, to correlate with the nomenclature used for the UKtc strain (McCrae and Faulkner-Valle 1981). The newly identified protein will be known as VP3.


GENOME : UKTC : PROTEINS : SA11 : GENOME
SP1
SP2
SP3
SP4
SP5
SP6
SP7
SP8
SP9
SP10
SP11
VP1
VP2
VP3
VP4
VP5
VP6
VP7
VP7c
VP8
VP9
vpr7
VP10
VP10c
VP11
VP11c
VP12
VP1
VP2
VP3
NS53
VP6
NS34
NS35
VP7
NS29
VP9
NS20
SP1
SP2
SP3
SP4
SP5
SP6
SP7
SP8
SP9
SP10
SP11

has been shown to reassort with virulence in NCDV X SA11 crosses (Offit et al 1986a) Haemaggiutinin activity and Protease enhanced plaque formation in UKtc X Rhesus MMU18006 strain (Kalica et al 1983), and restriction of growth of fastidious human strains in tissue culture (Greenberg et al 1981,1983a,b) Gene 6 has been identified as that encoding the subgroup-antigen (Human W, Wa and DS1 Greenberg et al 1983a,b; Human Wa and Bovine UKtc Kalica et al 1981a), while either Gene 8 or 9 depending on the strain encodes the neutralisation antigen VP7 (Greenberg et al 1983a,b, Kalica et al 1981a and Figure 2).

Two independent groups of Temperature Sensitive (ts) mutants of the UKtc virus have been isolated (Greenberg et al 1981, Faulkner-Valle et al 1982). Greenberg et al (1981,1983b) have used their mutants to "rescue" noncultivable human Rotaviruses, by the introduction of the bovine Gene 4. (Faulkner-Valle et al 1982,1983, and personal communication) have been able to demonstrate the existence of 10 of a possible 11 reassortant groups of a collection of more than 200 mutagen induced ts mutants. By reassortment with the genetically distinct porcine OSU virus the ts lesion of two of these groups has been located to a specific genome segment. In a further 6 cases a tentative assignment has been made. The ts mutants fall into two classes; efficiency of plaquing (EOP) mutants, which although they do not form foci in tissue culture, are still able to replicate at the non-permissive temperature, and EOP/yield mutants which show a markedly reduced virus yield at the non-permissive temperature. By performing up-shift and down-shift experiments and by studying protein and RNA synthesis at the non-permissive temperature it should be possible to elucidate the functions of some of the gene

products not yet characterised. Ts mutants will probably also be of value in development of live vaccine strains (Section 1(vi)) and in the detailed understanding of Rotavirus replication.

Rotaviruses have been isolated from a wide range of animal hosts (Flewett and Woode 1978) and there is therefore a very large gene pool present in the field. Due to the ability to undergo high-frequency reassortment there is the potential to rapidly generate new Rotavirus genotypes. Pedley and McCrae (1984) developed a rapid-screening assay based on the dot-blot methodology for surveying the genetic composition of Rotavirus isolates from cattle. Using information generated from such a survey it should be possible to determine whether this gene reassortment is significant in terms of the emergence of antigenic variants and possibly more virulent strains. This possibility needs to be taken into account in the development and use of vaccines (Clarke and McCrae 1981). See also addendum 2 for more details on gene reassortment.

GENOME REARRANGEMENTS

As described in section (1(ii)b) the typical genome pattern of the main Rotavirus group, the group A Rotaviruses, (see section 1(v) for description of the non-group A viruses) is composed of 11 segments migrating in the characteristic 1-2-3-4, 5-6, 7-8-9, 10-11 pattern. In recent years Rotavirus isolates have been found which although they are clearly group A viruses according to antigenic criteria, their genomes are unusual. These viruses lack one or more bands which are apparently replaced by other larger bands, likely to be concatameric forms of the absent one(s). By far the most

attention has been paid to isolates from chronically infected immunodeficient children; Pedley at al (1984) analysed the genomes of Rotaviruses excreted from two children suffering from protracted diarrhoea (PD) and severe combined immunodeficiency (SCID), in one case for 7 weeks and in the other for 8 months (in an acute infection virus is cleared from the body within 4-5 days). It was apparent from comparison of the genomes of sequential samples that additional bands were emerging, migrating between segments 1 and 7,8,9 and changing in quantity and pattern during the course of the infection. Northern blot analysis revealed that at least some of the additional bands contained sequences derived from normal RNA segments, almost always smaller ones, and must therefore have been the result of some form of concatermerisation, which was subsequently shown to be of a covalent nature. Analysis of the molecular weight of the new bands and the genes from which they were derived revealed that they were not simple integer multiplications of the genes. It was also shown by quantitation of silver-stained genome profiles that the appearance of new bands coincided with a relative decrease in the normal RNA segments from which they were derived (Pedley et al 1984).

An example of one such isolate which has been studied in detail was isolated from a calf and is known as Rotavirus 5244. This isolate lacks the smallest genome segment, gene 11 which is replaced by a larger segment known as "species X" migrating between segments 6 and 7. Cloning and sequencing of Sp11 from the UKtc strain and SpX from the 5244 isolate has revealed that SpX consists of two almost complete copies of Sp11 joined in a head to tail fashion. The first copy terminates at

nucleotide 615, and is followed by nucleotide 42 of the second copy which then continues to completion at the 3' end (Pocock 1987, Scott, Tarlow and McCrae personal communcation 1987).

Rotavirus isolates showing genome heterogeneity have also been isolated from cattle (Sabara et al 1982b) and monkeys (Pereira et al 1984). In the case of the monkey virus the heterogeneity was due to the coexistence of two subpopulations of virus within an isolate. However in a recent report (Hundley et al 1987) it was shown that virus isolates from immunodeficient children also consisted of several subpopulations of virus (from three isolations 12 electropherotypes were demonstrated) but in this case genomic rearrangements were also present. Analysis of isolates adapted to growth in tissue culture showed that of 7 rearranged bands, two were derived from segment 11, 1 from segment 10 and 3 from segment 8. In the case of segment 10 and 11 the original band was absent but in segment 8 the original band and the rearranged band were both present. By size analysis the extra bands were shown to be between approximately 1.5 and 1.8 times the size of the gene of origin and virus carrying one or more rearranged segments contained up to 1,800 base pairs of extra RNA (9.6% of the total genome) without any apparent change in virion shape or size. Interestingly the protein products of these viruses were very similar to each other and to the standard Bovine Rotavirus (BRV) and although rearrangements have been observed in segment 6, the subgroup antigen (Pedley et al 1984) most of the rearrangements involved genes encoding non-structural proteins. Rotaviruses with genome rearrangements have also been isolated from asymptomatic infections (Besselaar et al 1986, Thouless et al 1986, Pocock 1987) and in tissue culture during serial passage at

high multiplicity of infection (Hundley et al 1985). In this last case Hundley et al (1985) were able to isolate Rotavirus particles lacking genome segment 5 (encoding VP5, a structural protein) but with rearranged bands containing gene 5 sequences. These isolates which failed to produce the normal gene 5 protein on infection of tissue culture cells were still able to grow to a titer comparable with wild-type virus.

It is possible that this ability to undergo genome rearrangement represents a further mechanism of evolution in dsRNA viruses, in addition to the large potential for variation which already exists in their ability to undergo gene reassortment during mixed infections (see epidemiology section 1iv.c) and the accumulation of sequential point mutations (Follett and Desselberger 1983a). It has already been illustrated in tissue culture that human Rotaviuses carrying rearranged genomes are capable of undergoing gene reassortment with bovine Rotavirus (Allen and Desselberger 1985) further amplifying the potential for variation.

e) ROTAVIRUS ENZYME ACTIVITIES

Like other members of the Reoviridae, Rotavirions contain an RNA dependent RNA polymerase (Cohen 1977), and the RNA synthesised in vitro is a single stranded copy of the entire genome (Cohen and Dobos 1979). The polymerase was shown to be inactive in double shelled particles but active in cores produced by heat shock or treatment of virions with 1mM EDTA (which chelates the Ca^{2+} ions stabilising the outer shell (Cohen et al 1979). The properties of bovine and human RNA polymerase are similar (Spencer and Arias 1981). For optimum activity the polymerase requires all 4 ribonucleoside triphosphates, magnesium ions and a pH of 8.5. Activity is inhibited by sodium pyrophosphate but not by actinomycin D, rifampicin or α-amanitin (Cohen 1977, Spencer and Arias 1981), and in human viruses there appears to be a requirement for hydrolysable ATP, important for polymerisation and probably initiation and elongation of RNA molecules (Spencer and Arias 1981). The polypeptide(s) responsible for the RNA polymerase activity have not yet been identified. Candidates are obviously those proteins on the inner shell (eg. VP1-3) due to their proximity with the template RNA.

The structure of both the 5' and 3' termini of the human Rotavirus genome segments have been determined by Imai et al (1983), who were able to define a cap structure at the 5' termini. However, the enzyme responsible for this capping has not yet been identified.

The only other enzyme activity so far detected in Rotavirus particles is a poly(A)-polymerase found in a human virus (Gorziglia and Esparza 1981) and which could only be detected in

double-shelled particles. Since the viral mRNA is not polyadenylated the presence of the enzyme is difficult to explain and so its validity as a virion enzyme is unsubstantiated.

iii. ROTAVIRUS REPLICATION

The study of Rotaviruses isolated from humans and various animal sources has been severely hampered by their fastidious growth properties in tissue culture. Although the simian Rotavirus SA11 and the "O" agent were propagated in tissue culture in the 1960's (Malherbe and Strickland-Chomley 1967) before they were known to be associated with clinical illness, and bovine Rotavirus (BRV) was successfully grown in calf kidney cells only 2 years after its identification (Mebus et al 1971a) up until 1981 only a limited number of bovine and porcine strains (Woode et al 1974, McNulty et al 1967, Theil et al 1977) and human Rotavirus strain Wa (Drozdov et al 1977) could be propagated in tissue culture. A major breakthrough was made when it was discovered that pancreatic proteolytic enzymes enhanced viral replication (Babiuk et al 1977b, Theil et al 1977, Almeida et al 1978) and as a result it is now possible to sucessfully cultivate many Rotavirus strains in tissue culture using trypsin treatment to facilitate infection (Kutsuzawa et al 1982, Sato et al 1981). As a result of this adaptation to growth in tissue culture the Simian SA11 and the bovine UKtc Rotaviruses have been studied in detail.

The replication of the tissue culture adapted Rotaviruses takes approximately 12 hours under one-step growth conditions. At 37° C virus production begins 2 to 3 hours post-infection and the maximum level is reached by 12 hours (McCrae and Faulkner-Valle 1981). Protein synthesis is maximal 3-5 hours post

infection (McCrae and Faulkner-Valle 1981, Ericson et al 1982) and viral antigens can be detected by fluorescent antibody techniques by 4 to 6 hours (Estes et al 1979).

Rotavirus replication occurs in the cell cytoplasm, viral antigens are visible as granules, first around the nucleus and then throughout the cytoplasm. The speed and extent of infection of a cell culture by Rotavirus are enhanced by pre-activation with trypsin and the maintenance of trypsin in the medium throughout infection (Graham and Estes 1980). Synthesis of dsRNA is first observed by 3 to 4 hours post-infection and continues throughout the replication cycle (McCrae and Faulkner-Valle 1981).

VIRUS ENTRY

Studies on the early stages of infection suggested that virions enter the cell by endocytosis and are then sequestered into lysosomes (Petric et al 1981) similar to the situation with Reovirus (see Section 2). It is thought that uncoating occurs within lysosomes, followed by release of the sub-viral particles (SVPs) into the cytoplasm where replication takes place.

More recently Suzuki et al have studied the entry of human Rotavirus strain KUN into MA104 cells. It was found that this Rotavirus could enter the cell by endocytosis (in the absence of trypsin) but that no viral replication followed (Suzuki et al 1986). Two additional modes of entry which result in viral replication have been documented by this group; Firstly KUN Rotavirus may enter cells by direct penetration of the cell membrane (Suzuki et al 1985). Secondly in the presence of trypsin, virus particles have been seen to remain on the cell surface and expel their RNA from the core into the cytoplasm through radial spaces between the capsomers, and the cell

membrane pores formed after their attachment. This mode of entry, analogous to that used by the bacteriophage has not been previously observed in an animal virus system. These authors propose that as the nucleic acid leaves the virus core, it moves past the RNA transcriptase site(s) facilitating the synthesis of viral mRNA (Suzuki et al 1986). However, free dsRNA has never been seen in Reovirus infected cells (Silverstein and Dales 1978) and mRNA synthesis is thought to occur inside the sub-viral particles and so the significance of the above observations to Rotavirus replication requires further investigation.

MORPHOGENESIS

The first progeny virus particles are observed after an eclipse phase (2-3 hours post-infection) and consist of a dense nucleoid (25-35nm) with a surrounding layer of less dense material. They are formed near to granular cytoplasmic inclusions, which are thought to be an accumulation of newly synthesized viral protein and possibly viral RNA (Petrie et al 1982). Viral RNA is then packaged into 38nm particles known as "cores" within the viroplasm and then the outer capsid proteins are assembled around these. Studies using Tunicamycin have yielded conflicting evidence as to whether glycosylation of the outer shell neutralisation antigen VP7 is necessary for correct morphogenesis, Sabara et al (1982a) and Suzuki et al (1984) report that interference with glycosylation prevents correct assembly of the outer shell proteins with subsequent reduction in infectivity, while Petrie et al (1983) find that glycosylation specifically of VP7 is not required for normal viral morphogenesis. Shahrabadi and Lee (1986) have illustrated that calcium is necessary

for the addition of the outer protein coat; virus particles grown in the absense of calcium ions are poorly infectious and mainly single shelled (Section 1iia; Ca^{2+} stabilises the outer shell).

The progeny virus particles leave the cell by first budding though ribosome-free areas of the Rough Endoplasmic Reticulum (RER) into swollen vesicles; one of the most characteristic features of Rotavirus morphogenesis are the distended cisternae of the RER and the large vacuoles filled with virus particles. The virus particles become enveloped during their passage through the RER, and are expelled from the cell by exocytosis or rupture of the membrane.

Many studies of Rotavirus morphogenesis have been carried out both in tissue culture and in intestinal cells of infected animals (Chasey 1977, McNulty 1978, Saif et al 1978, Altenburg et al 1980, Esparza et al 1980, Petrie et al 1981, Suzuki et al 1981) and have resulted in the observations summarised above. In addition Chasey has identified 5 morphological types of virus particle which are thought to represent progressive stages of maturation (Chasey 1977).

Immunocytochemical electron microscopy has shown that the Rotavirus inner capsid proteins are synthesized throughout the cytoplasm and become concentrated in viroplasmic inclusions (Chasey 1980, Petrie et al 1982) while the outer capsid glycoprotein is synthesised by ribosomes in the RER, which suggests that the outer layer is acquired during viral budding into the cisternae of the RER. Further studies with monospecific antisera should reveal how Rotavirus assembly takes place (Petrie et al 1982).

REPLICATION OF THE ROTAVIRUS GENOME

The transcription and replication of the Rotavirus genome has not yet been studied in great detail. Current knowledge results from a limited number of studies in-vitro, with the endogenous RNA polymerase. Virus particles can be activated in-vitro by treatment with a protease or chelating agent, and then supplied with the substrates for RNA synthesis. Initial studies (Cohen1977) identified the transcription products from such a system as a complete single-stranded copy of the viral genome, which was active in a messenger dependent in-vitro translation system derived from rabbit reticulocytes. In vitro transcription of both SA11 (Mason et al 1980) and the Bovine UKtc virus have been studied , it is apparent that mRNA is identical to the plus sense strand of the dsRNA genome (Bernstein and Hruska 1981) a feature exploited by McCrae and McCorquodale (1982) in determining the gene coding assignments for the Uktc strain. These mRNA transcripts are not polyadenylated (Bernstein and Hruska 1981) but are translated more efficiently when in-vitro transcription is carried out in the presence of s-adenosyl methionine, indicating that the RNA transcripts may be capped (Mason et al 1980).

Recently Patton (1986) used sub-viral particles prepared from cells infected with SA11 to study aspects of Rotavirus replication. He found that the replication of the RNA appears to be assymetrical with synthesis of the negative sense strand depending on the presence of an mRNA template. It also appears that initiation of replication is dependent on protein synthesis but that elongation of the negative sense strand is not. He postulated that protein synthesis is necessary for the production of a ribonucleoprotein complex having replicase activity. The results obtained with this

system indicate that Rotavirus RNA synthesis probably follows the same pattern as Reovirus (Section 2), however these observations require confirmation in-vivo before any firm conclusions can be reached.

PROTEINS IN TRANSCRIPTION

The proteins involved in transcription and replication of the Rotavirus RNA have not yet been identified, although some have been shown to have properties which makes them likely candidates, such as the VP8 of Uktc (Baybutt and McCrae personal communication) and VP2 and NS31 of Bovine Rotavirus (Boyle and Holmes 1986) all of which have been shown to possess RNA binding properties (Section 1(ii)c). In the cell free system described above it was found that particles with replicase and transcriptase activity consist of the core proteins VP1 and VP2. Particles having replicase activity also contained smaller amounts of the inner shell protein VP6, and larger amounts of the non-structural protein NS34, relative to the transcriptase particles indicating a possible enzymic role for these proteins (Helmberger-Jones and Patton 1986, Patton 1986).

Sandino et al (1986) carried out a series of experiments with human Rotavirus: They took single shelled virus particles which were able to transcribe all 11 viral genes, and treated them with Calcium chloride to produce viral cores unable to transcribe. VP6 was the only protein removed by this treatment and reconstitution of these cores with VP6 from homologous or heterologous Rotavirus restored the RNA transcriptase activity and the normal profile of mRNA molecules was then produced. That transcriptase activity was restored by VP6 from a virus belonging to a different sub-group indicates that the capacity of VP6 to make transcription

possible is independent of the antigenic properties of the protein. Since purified VP6 alone does not possess any enzymic activities, it is thought either that VP6 contributes to an enzymic site on the inner virus shell, or more likely, that its presence in the core maintains the conformation of an enzymic site formed by one or more of the other constituents. The recent observations described above will hopefully lead to a fuller understanding of the regulation of Rotavirus RNA transcription and replication, and the proteins involved in these functions.

iv. ROTAVIRUS INFECTION

a) ROTAVIRUS SUBGROUPS AND SEROTYPES.

There are now known to be many different strains of Rotavirus. These can be classified according to the 2 criteria of Subgroup and Serotype.

SUBGROUPS

The two subgroups of Rotavirus have already been described briefly in section 1(ii)b. They are distiguished in non-neutralising assays such as Immune Adherence Haemagglutination assays (IAHA) and Enzyme-Linked Immunosorbent assays (ELISA), using post-infection sera to make such tests specific. The antigen recognised in these assays, and that used to define subgroups is the "subgroup antigen" VP6 located on the inner of the two capsid layers.

encoded by viral genome segment 6 (Smith et al 1980(SA11), Kalica et al 1981a, McCrae and McCorquodale 1982a(UKtc)). The correlation of subgroups with the "short" and "long" genome profiles described in section 1(ii)b is now known to be coincidental (Kalica et al 1981b). As these assays are non-neutralising, the antigen recognised is distinct from those defining serotypes.

Using IAHA and ELISA it has been possible to assign Human Rotavirus DS1, Bovine UKtc and NCDV, Porcine OSU, Simian SA11, Rhesus 1 and Rhesus 2 and the "O" agent to subgroup 1, while the Human strains D and Wa comprise Subgroup 2. The mouse EDIM virus has not yet been classified.

SEROTYPES

Serotypes of Rotavirus are defined on the basis of their neutralisation antigens and viruses are classified by means of a plaque reduction assay, which is able to distinguish between cultivable viruses isolated from bovine, canine, feline, simian and porcine sources (Hoshino et al 1981, Wyatt et al 1982). For viruses which grow poorly in tissue culture and are incapable of plaque formation, neutralisation of fluorescent cell-forming units has been used for serotyping (Flewett et al 1978, Thouless et al 1978, Zissis and Lambert 1978, Beards et al 1980, McNulty et al 1980). Greenberg and his colleagues (1982) have suceeded in serotypic characterisation of non-cultivable human Rotavius by gene reassortment methods where the ability of different Rotavirus isolates to undergo gene reassortment is exploited; by trasnfer of genes from a cultivable strain to a non-cultivable strain reassortant viruses are generated which are able to grow in tissue culture but at the same time retain the antigenic characterisitcs of the non-cultivable parent isolate.

More recently a radioimmunofocus assay has been developed for both cultivable and non-cultivable strains , which is reported to be more sensitive than plaque reduction assay (Liu et al 1984).

The viral protein responsible for eliciting neutralising antibodies and that detected in the assays described above is VP7, an outer shell glycoprotein (Section 1iic). This is encoded by genome segment 8 in the case of UKtc (McCrae and McCorquodale 1982), and species 9 in the case of SA11 (Kantharidis et al 1983). Until recently it was thought that VP7 was the sole determinant of neutralisation specificity, however studies by Hoshino et al (1985) on a human Rotavirus strain indicate that VP3 (equivalent to the UKtc VP4 and encoded by gene 4 in both strains) located on the outer capsid shell may also play a part in neutralising specificities. If this is true then the involvement of this protein needs to be considered in the development of vaccine strains (Section 1(iv)).

There are now known to be at least 4 and possibly 5 serotypes of human Rotavirus (Matsuno et al 1985, Albert et al 1987), 3 of bovine Rotavirus (Snodgrass et al 1984, Brussow et al 1987) and 3 of avian Rotavirus (McNulty et al 1980). Clearly a detailed knoweledge of Rotavirus serotypes and their global distribution is of great importance in the development of anti-viral vaccines, since immunity to one serotype will not automatically confer immunity to a second.

Rotaviruses of mammalian and avian origin share antigenic determinants other than the subgroup and neutralistion antigens. These common determinants are located on the inner shell and are specific to Rotavirus, within the family Reoviridae (Woode et al 1976, McNulty et al 1979). The existence of these common antigens and the discovery that antisera against single-shelled Rotavirus

particles will cross react in serological tests (Bridger 1978) means that the cultivatable animal Rotaviruses and antisera raised against them, and also monoclonal antibody reagents specific for VP6 (Greenberg et al 1983, and addendum 1) can be used for the detection of human Rotavirus infection at the level of faeces and serum (Matsuno et al 1977a, Schoub et al 1977, Bishai et al 1979, Brade and Schmidt 1979) therefore providing a useful diagnostic tool.

b) PATHOLOGY AND IMMUNITY

PATHOLOGY

Most knowledge of the pathology of Rotavirus infection has been gained from study of natural and experimental infections in animal such as calves, pigs and lambs although recently it has been possible to study a limited number of mucosal biopsies taken from children. Since the pathology seen in all cases is similar it is possible to make several generalisations.

Immunofluorescence staining has shown that Rotavirus replicates mainly and usually only in the columnar epithelial cells lining the small intestine (Mebus et al 1971b,1977, McNulty et al 1976, Wyatt et al 1976, Mebus and Newman 1977, Snodgrass et al 1977a, Theil et al 1978a). These cells differentiate continuously; cell division occurs in the crypts, from where cells move up the sides of the villi elongating as they do so and becoming columnar, gaining a brush border and numerous enzymic activities. The cells are fully differentiated by the time they are between half and a third of the way along the villi and their life is then limited to only a few days, before they reach the villus tips and fall off. Only fully differentiated cells are susceptible to infection by Rotavirus.

During Rotavirus infection many changes in sub-cellular morphology are observed; The appearance of masses of convoluted smooth membranes, (which has also been seen in other infections, such as mouse hepatitis virus , rubella and St Louis encephalitis) is thought to be due to an alteration in cell metabolism rather than the accumulation of viral products (Estes et al 1983). Late in infection changes in the nucleus are also seen, such as separation of the nuclear envelope and the accumulation of virus particles between the lamellae (Estes et al 1983). Filaments seen in the cytoplasm are occasionally seen in the nucleus accompanied by viroplasm (accumulation of viral proteins). Virus particles have also been observed in mitochondria (Altenburg et al 1980). The damage caused to cellular integrity by virus replication is reflected biochemically; synthesis of cellular DNA, RNA and protein are all inhibited (Carpio et al 1981, McCrae and Faulkner-Valle 1981, Ericson et al 1982) with the end result that cells are destroyed and shed from the villus surface prematurely (the virus content of faeces is mainly due to the presence of cells containing virus). The villi then appear shortened and are covered in immature cuboidal epithelial cells which have reduced levels of disaccharidases (including lactase), and an immature glucose coupled sodium transport mechanism (Davidson et al 1977, Davidson and Barnes 1979). This leaves undigested lactose (from milk) in the intestine where it has an osmotic effect, preventing the uptake of water and together with bacterial degradation of the lactose in the bowel leads to diarrhoea and subsequent dehydration (Flewett 1977). Because only the differentiated epithelial cells are susceptible to infection (virus particles or antigens have never been seen in the crypts) the rapid shedding of infected cells from the villus tips is thought to limit the duration of infection (Mebus et

al 1971) and the villi return to their normal state within three to four weeks. It is possible that undifferentiated cells are not susceptible to Rotavirus infection because they lack receptors for the virus (Holmes et al 1976). Although it is mainly the cells of the small intestine which become infected immunofluorescence studies in mice, humans and lambs have shown that epithelial cells in the colon can also be susceptible. This is not true of calves and is only rarely seen in pigs (Banfield et al 1968, Hamilton et al 1976, Snodgrass et al 1977, Theil et al 1978).

IMMUNITY

Anti Rotavirus serum antibody in both animals and man is maintained at a high level. For instance Flewett and Woode (1978) estimate that 90% of children have specific antibodies by the age of 6. Similarly 100% of 59 herds of cattle in the UK and 75% of 30 Canadian herds were also seropositive for Rotavirus (Flewett and Woode 1978).

Although IgG and IgM serum antibodies can be detected in children and adults it has been shown that at least in young animals maternal serum antibody obtained transplacentally does not afford any protection against infection (Woode et al 1975, Snodgrass and Wells 1976, McNulty 1978). The same is true of human neonates; it has been found that children and adults with serum antibody indicating prior infections are not fully resistant to Rotavirus infection.

The important factor in Rotavirus immunity appears to be secretory IgA antibodies which are present both in the intestine and in the colostrum and milk of animals and man. It has been demonstrated that maternal colostral antibody is protective in

calves, lambs, piglets and humans (Woode et al 1975, Snodgrass and Wells 1976, Bohl 1979, McClean and Holmes 1981) and such antibodies provided they are neutralising appear to protect heterologous species when given orally (Leece et al 1976, Snodgrass and Wells 1978, Bartz et al 1980). In bovine colostrum upto 14-28 days post-partum anti-Rotavirus antibodies are a mixture of IgG and IgA, IgG becoming predominant (Corthier and Franz 1981, Hess and Bachmann 1981). Almost all the anti-Rotavirus immunoglobulin in human milk and colostrum is IgA (Yolken et al 1978, Cukor et al 1979, McClean and Holmes 1980) and although breast-feeding does not prevent infants getting Rotavirus gastroenteritis (Weinberg et al 1984) it does seem to moderate the course of infection, symptoms are reduced in both duration and severity. Antibodies present in colostrum are at their maximum almost immediately after parturition, and thereafter decline rapidly (Woode et al 1975, Thouless et al 1977, McClean and Holmes 1980). Infections in suckling animals are delayed rather than prevented completely and the severity of any infection contracted during this decline depends on the antibody titer of the colostrum and the volume taken by the young animal (Snodgrass and Wells 1978).

Because of the absence of a good experimental model very little is known about the development of intestinal immunity during human Rotavirus infections (animals remain susceptible to infection for a much shorter period than human infants (Section 1(iv)a). Recently work has been carried out to ascertain the importance of coproantibodies during infantile Rotavirus infections (Coulson et al personnal communication). These were first reported by Sonza and Holmes (1980) who detected specific IgA, IgM and IgG in the faeces of 4 children and 1 adult, the peak titers occurring between 10 and 30 days after the appearance of symptoms, and Grauballe et al

(1981) reported the persistence of coproantibody for upto 7 months after infection. Since this antibody is presumably that in closest proximity with the Rotavirus and the infected cells, it could be extremely important in determining the severity and/or longevity of an infection (Hjelt et al 1987, Bernstein et al 1986). Much more investigation is required before any firm conclusions can be reached.

Very little is known about the role played by the cell mediated response to Rotavirus infection. The possibility that the product of gene 4 is involved is currently under investigation (Jenkins and McCrae personal communication).

c) EPIDEMIOLOGY OF ROTAVIRUS INFECTIONS

MODE OF TRANSMISSION

Because Rotaviruses infect and cause the loss of intestinal epithelial cells they are often excreted in the faeces of infected individuals in huge amounts. In humans estimates of upto 1 X 10^9 and 1 X 10^{10} virus particles per gram have been made (Davidson et al 1975, Chrystie et al 1978) and because of the high calcium content present in the faeces of young unweaned animals, it is thought that Rotavirus infectivity remains stable for some time (Shirley et al 1981). It is generally accepted that most Rotavirus infections are acquired through direct contact with infected individuals or an infected environment. For example in hospital nurseries Rotavirus infections are more common in babies kept in a communal situation than those kept with their mothers (Bishop et al 1979). It is because of this mode of transmission that the intensive

rearing of young animals is especially vulnerable to Rotavirus infection and once infection has gained entry into an animal population its rapid dissemination is virtually impossible to prevent.

Rotavirus particles are very stable entities, they are capable of survival in water and are resistant to chlorination (Snodgrass and Herring 1977, Tan and Schnagl 1981) suggesting that Rotavirus infection can be contracted even from a clean water supply. Indeed Lycke et al (1978) reported over 3000 human Rotavirus infections in a Swedish town as a result of the freshwater supply being contaminated with sewage effluent. In developing countries where sanitation is poor, sewage and water are almost certainly the source of a great many infections.

Although Rotavirus infections occur predominantly in the young of human and animals, infections can also occur throughout life although often asymptomatically (McNulty 1978, Holmes 1979). In animals the most severe infections are those acquired during the first few weeks of life, while in humans these early infections are frequently mild or asymptomatic (Cameron et al 1978, Chrystie et al 1978) and the most serious infections occurr between 6 and 24 months of age (Bryden et al 1975, Davidson et al 1975). The number of Rotavirus infections in adults is unclear, since many incidents of diarrhoea remain unreported. However infections are frequently reported in parents, hospital staff and others in contact with paediatric patients (von Bonsdoff et al 1976, Kim et al 1977, Rodrigues et al 1979, Wenman et al 1979). Epidemics are also documented among groups of elderly people (Cubitt and Holzel 1980, Harvorstud and Orstavik 1980).

EVIDENCE FOR ANTIGENIC VARIATION (DRIFT AND SHIFT) IN ROTAVIRUS INFECTIONS

i. ANTIGENIC SHIFT

It is now known that children suffer successive Rotavirus infections (Sack et al 1980, Wyatt et al 1979a) and they are often caused by viruses of different subgroups (Fonteyne et al 1978, Rodrigues et al 1978). Gel electrophoresis has been widely applied in recent years to follow successive Rotavirus infections in groups of children (Espejo et al 1980b, Rodger et al 1981, Chiba et al 1984, Follett et al 1984, Dolan et al 1985, Gomez et al 1986). This approach is based on the observation that Rotavirus sub-groups 1 and 2 are easily distinguishable by their "short" and "long" RNA profiles respectively (Section 1(ii)b), and it has become apparent that several different Rotaviruses can co-circulate in a population at any one time (Espejo et al 1980b, Schnagl et al 1981, Follett and Desselberger 1983,1983a, Follett et al 1984, Tam et al 1986, Hundley et al 1987). In a recent study in Sweden, it was found that over a period of 1 year, the circulating "pool" of Rotavirus changed from one where subgroup 1 and 2 co-circulated to one where subgroup 2 was predominant (Unhoo and Svensson 1986). In addition, 3 electrophoretic variants of subgroup 2 were detected (by PAGE analysis) and one of thee seemed to be of higher virulence than the other two. The shift from viruses of one subgroup to another over a short period of time is indicative of the possible occurrence of antigenic shift such as that documented for influenza virus. Such a shift could occur in the Rotavirus population by exchange of genome segments, and the existence of a diverse gene pool has been demonstrated (Espejo et al 1980,

Schnagl et al 1981, Clarke and McCrae 1981a,1982, Tam et al 1986). The potential for such reassortment events exists but its exact importance in the variation of Rotavirus antigenicity and virulence has yet to be determined.

ii. ANTIGENIC DRIFT

The appearance of a more virulent strain of Rotavirus without any apparent change in electropherotype may be indicative of antigenic drift. Coulson et al (1985) have found that 2 rotaviruses of the same electropherotype isolated from babies in a Melborne hospital only three months apart are antigenically distinct. It is known that RNA viruses have a high rate of mutation, attributable mainly to the absence of any RNA proof-reading and repair system in the infected cell (Holland et al 1982). In 1982 Street et al suggested that variation among Rotavirus strains occurs by a process involving both antigenic drift and shift. Using hybridisation analysis of RNA from human Rotaviruses collected in New Zealand between 1975 and 1980 they were able to show that electrophoretic variations were accompanied by sequence diversity (Street et al 1982).

Despite the usefulness of RNA electropherotyping in wholesale appraisal of the circulating Rotavirus population this method cannot be used to predict antigenic characteristics or virulence of the isolates studied. The correlation between subgroup and "long" and "short" profile is coincidental (Section 1(ii)b). In addition there appears to be no relationship between the sequence of a gene and its electrophoretic mobility (Clark and McCrae 1982); RNAs of very diverse sequence can migrate to the same position

in PAGE. However despite these limitations, electropherotyping remains a useful tool for the initial screening of Rotavirus populations particularly in primitive laboratory conditions.

v. THE ATYPICAL OR NON GROUP A ROTAVIRUSES

So far the description of Rotaviruses presented has referred exclusively to the "typical" or "group A" Rotaviruses. These viruses which have been isolated from a wide variety of animal species possess a common "group antigen" which is located on the inner of the two capsid layers, and is therefore only exposed in single-shelled particles, (Woode et al 1976) unlike the type-specific or neutralising antigen which is located on the outer layer (Bridger 1978). Between 1979 and 1982 several reports were published of viruses from birds and pigs which resembled Rotaviruses by morphological criteria, but which did not cross react with antisera to this "group antigen" by immunofluorescence (Bridger 1980, Saif et al 1980, McNulty et al 1981, Bridger et al 1982). Since then it has become apparent that there are several groups of Rotaviruses. The first group to be identified is now known as the "Group A" or "typical" Rotaviruses while those groups which followed are called "groups B through E" or "Atypical". The number of such groups each with its own group antigen continues to increase and currently stands at 4 (Pedley et al 1986) although the possibility that a further two groups exists is being investigated (McCrae, personal communication).

Atypical Rotaviruses have to date been identified in pigs (Bridger et al 1982, Pedley et al 1983,1986, Chasey and Davis 1984, Chasey et al 1986) chickens (Pedley et al 1986,

McNulty et al 1981) rats (Vonderfecht et al 1984) cattle (Chasey and Davis 1984) humans (Hung et al 1984, Bridger et al 1986) and lambs (Chasey and Banks 1984,1986).

The importance of the atypical Rotaviruses from an epidemiological point of view is not yet clear. Electrophoresis of their RNA genome shows a pattern distinct from that described for the group A viruses, the most obvious change being the absence of the 7-8-9 tight triplet of bands (Pedley et al 1983). It has been shown by terminal fingerprint analysis that representatives of the 5 Rotavirus groups, A,B, C (Pedley et al 1983), D and E (Pedley et al 1986) do not carry any genome segments in common with each other. It is therefore likely that they will represent distinct gene pools and will be unable to reassort with each other. Greenberg(1987) has tried to make reassortants between Group A mammalian and avian viruses but was unsuccessful and therefore maintains that the probability of viruses from different groups undergoing reassortment to be very unlikely as they are even less closely related than the two Group A viruses. However if it is the case that viruses from different groups can interact then the implication for epidemiology and vaccine development are far reaching, since the potential antigenic variability of the Rotaviruses as a whole will be greatly amplified. It has recently been reported that in lambs, the incidence of atypical Rotavirus infection is becoming predominant over typical infections (Chasey and Banks 1984) however if this is a real shift or merely a perceived one has yet to be clarified; since there is no serological test for the atypical Rotaviruses and many diagnostic tests use ELISA (assays in which the non-group

A viruses are not detected) it is probable that they had been circulating for some time before their discovery, and that their apparent increase is due to new awareness.

Very little is known about the molecular biology of the atypical Rotaviruses because of their very fastidious nature. Until recently with the exception of the Group D viruses they could not be propagated in tissue culture and so all analysis was carried out on viral RNA extracted from faeces which also contains numerous other nucleic acids. Recently the porcine Group C Rotavirus was adapted to growth in tissue culture (Terrett and Saif 1987) a developement which will greatly facilitate its molecular analysis. As yet the group B and E viruses are not cultivable.

Morphologically the atypical viruses resemble the typical Rotaviruses although some differences are now becoming apparent. For example, thin walled featureless particles of 48 to 52nm have been seen in a non-group A virus from pigs (Bridger et al 1982, Theil et al 1985) and humans (Hung et al 1983), similar 'core-like' particles are seen only after chemical treatment of the group A viruses. The disease state caused by the atypical viruses is identical to that for the typical viruses but virus particles appear to be excreted in smaller quantities (Bridger 1980). Similarly, although replication takes place in the same intestinal cells (McNulty et al 1981, Vonderfecht et al 1984, Theil et al 1985, Chasey et al 1986, Chasey and Banks 1986) the pathology of the atypical infection differs. Syncytia have been observed in experimental infections with Group B viruses (Bridger 1987) whereas Group A viruses do not produce syncytia in infected intestinal or tissue culture cells. Syncytia have not yet been

observed in infections with group D atypical Rotavirus (McNulty 1987) and the histopathology of group C infections has not yet been examined.

THE CHINESE "ADRV" ROTAVIRUS

Of the atypical Rotaviruses isolated to date the Chinese Group B virus has commanded the most attention. In late 1982 and early 1983, two epidemics of acute diarrhoea occurred in China, affecting over 12,000 adults. Prior to this, Rotavirus infection had only been associated in epidemic proportions with young children, but the virus isolated from stools resembled a Rotavirus and contained 11 species of dsRNA (Hung et al 1984). On serological examination, this virus which is known as ADRV (Adult Diarrhoea Rotavirus) was found to lack the Group antigen of typical Rotaviruses, and its genome profile lacked the 7,8,9 genome segment triplet. Genome profile analysis of ADRV suggested either that it belonged to the Group E atypical viruses or that it constituted a new group. Serological and nucleic acid hybridisation studies revealed that ADRV belongs to the Group B viruses (Chen et al 1985), and illustrates that atypical Rotaviruses should not be classified solely on the criterion of electropherotype.

The use of counterimmunoelectrophoresis (CEE) to detect ADRV antibodies revealed that infection with ADRV is widespread but maintained at low levels in China, and also in Hong-Kong and Australia where symptomatic infections have not yet been reported. In an area which had experienced an ADRV epidemic

the rate of seropositivity was twice as high as in a non-epidemic area, and levels of antibody were similar between individuals who had experienced symptoms of the infection and those who had not (Hung 1985). A low level of antibody to ADRV was also found in immunoglobulin pools in the UK, North America and Japan (Padley et al 1983, Hung et al 1985, Brown et al 1987, Nakata 1987) indicating that a large number of people are potentially susceptible to this virus. Recently group C viruses which were previously found only in animals have been isolated from humans in Australia, Brazil and the UK (Bridger et al 1986). The finding that prevalence of antibody to group C viruses in human sera and immunoglobulin pools from 6 different countries is low indicates that the group C as well as the group B viruses could be of great epidemiological importance in the future. Group B and C (in addition to Group A) viruses are therefore capable of infecting both pigs and man which indicates that they may be zoonotic infections. If this is so then the development of anti-Rotavirus vaccines must take into account antigens of all three of these groups, otherwise selective pressure may undermine any immunisation program instigated.

vi. TOWARDS AN EFFECTIVE ANTI-ROTAVIRUS VACCINE

Due to the large losses caused by Rotavirus infection (see Section 1ia) in terms of both infant mortality and economic loss, the development of an anti-Rotavirus vaccine has become a priority. A survey of cholera and Rotavirus infection in children in developing counties reached the conclusion that improvements in water supplies and standards of hygiene would be of little benefit, and control of Rotavirus infections would ultimately depend upon the use of an effective vaccine (deZoysa and Feacham 1985).

There are several possible approaches to the developement of a Rotavirus vaccine, and the one which appears most promising and is the most advanced in clinical evaluation follows the "Jennerian" approach, using a related virus from a different host as the vaccine strain. This is possible in the case of Rotaviruses, since as previously stated, viruses from different animal hosts share a common antigen, VP6 on the inner of the capsid shells (Flewett et al 1974). Evidence that immunisation could confer protection against challenge with a heterotypic virus was provided by Wyatt et al (1979b,c) who inoculated calves in utero with NCDV (a bovine Rotavirus) and then challenged at birth with human Rotavirus type 1. Inoculated calves were protected against the challenge, while most of the control calves were susceptible and developed diarrhoea (Wyatt et al (1979b,c). These calves were subsequently shown to have neutralising antibodies to human Rotavirus serotypes 1,2 and 3 at birth (Wyatt et al 1983). Bovine Rotavirusa has also been shown to protect colostrum deprived piglets against infection by human

Rotavirus (Zissis et al 1983). Since these first encouraging results two candidate vaccine strains have been developed and reached the stage of clinical trial.

BOVINE RIT 4237 VACCINE

This candidate vaccine strain is a cold adapted mutant of Bovine NCDV (Lincoln strain). It was attenuated by passing 147 times in bovine kidney cells, and 7 times in African Green Monkey cells. Three major clinical trials have been carried out: Two of these which were carried out in Finland gave very promising results. In the first a single vaccine dose conferred protection against "clinically significant" diarrhoea (of greater than 24 hours duration) in 88% of 178 infants aged between 8 and 11 months, and in the second study vaccine administered in two doses to 331 infants aged between 6 and 12 months, gave a clinical protection rate of 82% which corresponded well with seroconversion. Even infants without seroconversion experienced less diarrhoea than infants in the control group, confirming that immunity to Rotavirus infection is mediated by factors other than serum immunoglobulin (Vesikari et al 1983,1984,1985).

Following these sucessful trials, there were still a number of unanswered questions; How would maternal antibody (in breastfed babies) affect the vaccine? How effective would it be in developing countries? and would it react with other vaccines such as Polio virus when given in combination? The first two of these questions have been answered by a clinical trial carried out in Rwanda during 1983 and 1984 (DeMol et al 1986). 260 infants aged between 3 and 8 months, from a rural community were given either

vaccine or a placebo following breastfeeding and were monitored until 1985, with regard to episodes of diarrhoea, virus shedding and serum antibody. The results of this trial were very disappointing; there was no significant difference between the two groups in any of the three criteria listed above. The quality of the vaccine used was not questionable and so it appears that RIT 4237 is unsuitable for use in developing countries. It is thought that the maternal antibodies acquired through breastfeeding may have neutralised the effect of the vaccine or that the high frequency of enteric infections in this area may have interferred with the immune response (DeMol 1986). The third question above has also been answered: Vodopija et al (1986) found that when RIT4237 was administered with Polio virus types 1 and 3, the antibody response to RIT4237 was significantly reduced while that to Polio was unaffected. It appears that at the doses used in these trials vaccine RIT4237 is not effective.

RHESUS ROTAVIRUS (RRV-1) VACCINE

This second candidate vaccine was derived from the stool of a 3.5 month old Rhesus monkey suffering from diarrhea, passaged 9 times in primary or secondary monkey kidney cells, and 7 times in DBSFRhL-2 cells. The cell line was developed specifically as a potential substrate for vaccine production (Wallace et al 1973) since adventitious agents are often found in primary monkey kidney cell cultures, (indeed the human Rotavirus strain Wa which was adapted to growth in tissue culture and then given to human volunteers was subsequently found to be contaminated with a Simian foamy virus (Flewett 1986)). The

ability of this strain known as MMU18006 to grow in these cells together with the fact that it has never been isolated from humans under natural circumstances and that it is antigenically similar (and possibly identical) to human Rotavirus Serotype 3, made it a suitable choice for clinical trials. Trials have been carried out in volunteers in a range of age groups and the results are summarised. Vaccine was given orally at a dose of 1×10^5 to 1×10^6 PFU with a sodium bicarbonate buffer to prevent inactivation of the virus by stomach acid, and vaccinees were monitored both for effects due to the virus (eg. stool formation, virus shedding) and for other symptoms such as rhinorrhea. Vaccinees experienced at least a four-fold rise in neutralising antibody titer, and in older infants (8 to 61 months) the only adverse reaction to the vaccine was an increased incidence of rhinorrhea and virus shedding (Anderson et al 1986). In a further trial, 94% of 31 adults and 86% of 57 children (aged 4 months to 12 years) developed a serologic response to Rotavirus (Kapikian et al 1986). However, when RRV-1 was compared with RIT4237 in children of 6 to 8 months, the RRV-1 vaccine induced a fever (>38°c) in 64% (16 of 25) vacinees, occuring on days 3 and 4 post-vaccination, while RIT4237 only induced fever in 17% (4 of 24) vaccinees. Because of these adverse reactions RRV in its present form is not suitable for large scale administration to children under the age of 8 months. It is however significantly more antigenic that RIT4237 (illiciting a neutralising antibody response in 81% of vaccinees compared to 45% of those receiving RIT4237, and causing virus shedding in 81% of cases compared to 21% in RIT4237) (Vesikari et al 1986). A lower dose of RRV-1 (1×10^3 to 1×10^4 PFU) has since been tested in Venezuelan children aged between 4 and 10 months, and was found to be non-reactogenic (Perez-Schael et al

1987);82% of children vaccinated with 10^4PFU and 59% vaccinated with 10^3 PFU developed anti-Rotavirus antibody. The adverse reactions in the Finnish children may be explained by the observation that American and Venezuelan children (4-12 months of age) have significantly higher prevaccination serum antibody titers than the Finnish children (6-8 months of age Kapikian et al 1986). It may be that pre-existing antibody affects the clinical response without altering the immunogenicity of the vaccine. This observation becomes important when vaccination of younger children is considered who may have high levels of maternal antibody. It is predicted that in developing countries it will be necessary to vaccinate children within 2 months of birth, at a time when they appear to have a resistance to Rotavirus illness so that most infections are subclinical. How this resistance is mediated is unknown,and it is therefore necessary to use a dose of RRV-1 that will be sufficiently infectious to stimulate an asymptomatic infection in the presence of the passively acquired maternal antibody. Of 49 Venezuelan infants (1 to 4 months) given 1 X 10^4 PFU, 75% developed anti-Rotavirus antibody and no adverse reactions were recorded. Further trials are underway in the US and other countries. The question remains whether immunisation with a single heterologous vaccine strain will afford protection against all four human Rotavirus serotypes. Several points indicate that vaccines such as that outlined above will do this; the RIT4237 vaccine induced protection against subsequent infection by human serotype 1 (Vesikari et al 1985), it is thought that children usually undergo only one severe Rotavirus infection, and naturally occuring

subclinical infections of neonates can protect them for upto three years from severe Rotavirus infection by viruses of more than one serotype.

A second approach to vaccine development exploits the ability of Rotaviruses to undergo gene reassortment. For example, Midthun et al (1985,1986) have prepared single gene substitution reassortants for human serotypes 1,2 and 4, which have 10 RRV-1 gene segments and the human gene encoding the neutralisation antigen, VP7. Similar reassortants between the bovine UKtc strain and the four human serotypes have also been prepared (Midthun et al 1985,1986). To date only one such candidate vaccine has been tried in volunteers. Clark et al (1986) inoculated human adults with NCDV X human Wa reassortant viruses, which contained Wa segment 9 and the serotype 1 neutralisation phenotype. Very little seroconversion has been detected, and it is not yet known if the vaccinees will be protected against subsequent challenge, or how children will respond to the vaccine.

Finally the possibility of developing a subunit vaccine is currently under investigation. The neutralisation antigen of the Bovine (UKtc) Rotavirus has been cloned and expressed to high levels in E.coli (McCrae and McCorquodale 1987). As described in section (1(iv)b) it appears that the important immunoglobulin in Rotavirus immunity is secretory IgA, produced at the site of infection. By administering this expressed protein orally to the cow it is predicted that an ScIgA antibody response will be induced in the intestine and that calves will be protected in early life by Sc.IgA received via milk and colostrum. To date this protein has been used to immunise one gnotobiotic

calf, by oral administration of a lysate of bacteria expressing the protein. Unfortunately the calf was not protected against a subsequent challenge with Rotavirus (Bridger and McCrae, personal communication) although when the same protein in a purified form was inoculated sub-cutaneously into rabbits, neutralising serum antibody could be detected in plaque reduction assays.

2. REPLICATION OF REOVIRUS

A. THE FAMILY REOVIRIDAE

The Rotaviruses are one of several virus genera forming the family "Reoviridae". The other members are the Reoviruses themselves (the Orthoreoviruses), the Orbiviruses (eg.Bluetongue virus: Verwoerd 1969), the Cypoviruses (eg. Cytoplasmic polyhedrosis virus: Miura et al 1968) the Phytoreoviruses (eg. Wound tumour virus: Gomatos and Tamm 1963) and the Fijiviruses of plants and insects. These viruses of which the Reoviruses are the best understood, have several features in common. They all have a double-stranded RNA genome containing between 10 and 12 individual and unique segments, and all of them have an icosahedral virus particle 70nm in diameter composed of two concentric capsomer shells (although in the case of the Cypoviruses and the Fijiviruses the existence of a second capsid layer has not yet been confirmed).

Following infection of susceptible cells, members of this group remain partially uncoated and transcribe the dsRNA genome into translationally competant mRNAs using enzymes present in the viral core.

Because of the inclusion of the Rotaviruses in this family, it has been assumed that they will be similar to Reovirus in many aspects of their replication. The structure and replication of the RNA genome of Reovirus has been well documented, while that of the Rotaviruses remains largely unstudied. The purpose of the work described in this thesis is to determine molecular details of Rotavirus RNA replication. Since all of the experimental work has been based on the assumption that Rotavirus replication will be similar to that of Reovirus, a detailed description of the replication of Reovirus is appropriate.

B. REOVIRUS RNA REPLICATION: AN OVERVIEW

The RNA of Reovirus is replicated in a fully non-conservative fashion; this mechanism was first proposed in 1971 by Schonberg et al, who found that when Reovirus particles infect cells, the dsRNA genes are first transcribed into single-stranded plus-sense (coding) transcripts that initially function as messenger RNA. Later in infection these mRNA molecules are assembled into sets of 10, and are then copied into minus-strands with which they remain associated to form the progeny genome. The synthesis of the two complementary strands of the Reovirus genome is therefore temporally separated, plus-sense

RNA being produced earlier in infection and then used as template for the production of minus-sense RNA which occurs later in infection (Acs et al 1971, Schonberg et al 1971, Silverstein et al 1976). Since parental RNA remains inside the infecting particles and does not appear in the progeny this mode of replication is termed "fully non-conservative". The replication strategy used by Reovirus is summarised in Figure 2a (Reproduced and modified from Joklik 1981).

C. THE REOVIRUS PARTICLE

The Reovirus particle is icosahedral and consists of two concentric protein shells, the inner of which contains the viral RNA. The protein composition of Reovirions was first examined by Loh and Shatkin (1968) and Smith et al (1969) who developed the nomenclature a modified form of which is used today (McCrae and Joklik 1978). There are a total of 12 virus specific proteins, encoded by the 10 dsRNA genes, of these 9 are found in the virus particle, and two are non-structural. With the exception of gene S1 all the RNAs are thought to be mono-cistronic. S1 contains two open reading frames, the first encodes σ1 and the second σ1s a minor protein whose function or location has not yet been determined (Ernst and Shatkin 1985, Jacobs and Samuel 1985). Table 1 summarises the available information about the Reovirus proteins (McCrae 1987).

To date, the known functions of the Reovirus proteins are as follows;

FIGURE 2a: A SCHEMATIC REPRESENTATION OF THE REOVIRUS REPLICATION STRATEGY.

(Reproduced and modified from Joklik 1981)

After infection of permissive cells double-shelled Reovirus particles uncoat to form the parental sub-viral particles in which the transcriptase is active. mRNA copies of the 10 genes are transcribed (early mRNA) which has two functions: Some is translated to yield viral proteins, and some is packaged at the rate of one molecule of each segment per particle with the newly synthesised protein to form immature virus particles. These progeny particles are then capable of transcribing more mRNA which in turn may be packaged into immature virus particles. Transcriptional activity ceases when the outer coat of the virion is added thus inactivating the polymerase.

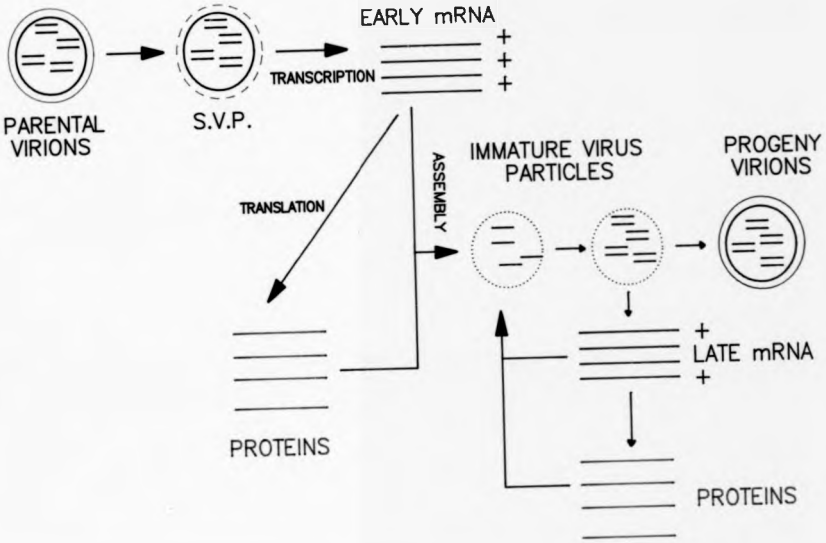

EARLY mRNA
+
+
+
TRANSCRIPTION
PARENTAL VIRIONS
S.V.P.
TRANSLATION
ASSEMBLY
IMMATURE VIRUS PARTICLES
PROGENY VIRIONS
+
LATE mRNA
+
PROTEINS
PROTEINS

TABLE 1: SUMMARY OF AVAILABLE INFORMATION ON REOVIRUS PROTEINS.

(Reproduced from McCrae 1987).

PROTEIN SPECIES	ENCODING GENE	APPROXIMATE MOLECULAR WEIGHT	APPROXIMATE % OF TOTAL PROTEIN	LOCATION WITHIN VIRION	POST-TRANSLATIONAL MODIFICATION
	STRUCTURAL				
λ 1	L3	155,000	15	CORE	NO
λ 2	L2	140,000	11	CORE	PROTEOLYTIC CLEAVAGE
λ 3	L1	135,000	<2	CORE	NO
μ 1	M2	76,000	2	CORE	CLEAVAGE TO μ 1C
μ 1C	M2	68,000	35	OUTER SHELL	GLYCOSYLATION? PHOSPHORYLATION? ADP RIBOSYLATION
μ 2	M1	70,000	<2	CORE/ OUTER SHELL	NO
σ 1	S1	42,000	1	OUTER SHELL	NO
σ 2	S2	38,000	7	CORE	NO
σ 3	S4	34,000	28	OUTER SHELL	ADP RIBOSYLATION
	NON- STRUCTURAL				
μ NS	M3	75,000	-	-	PROTEOLYTIC CLEAVAGE
σ NS	S3	36,000	-	-	NO
ρ 14	S1	14,000	-	-	NO

Of the 5 proteins definitely present in the viral core (λ1,λ2,λ3,μ1,σ2) the functions of only two have been determined. λ2 is thought to be the sole constituent of the 12 core projections, through which single-stranded transcripts of the genes are extruded after synthesis (Gilles et al 1971), and it is therefore postulated that the transcriptase catalytic sites are at the base of the spikes, facilitating this process. Each spike is composed of a pentamer of λ2 (Ralph et al 1980) and it is known from studies with monoclonal IgG's directed against λ2 that the spikes project through the outer capsid shell and are exposed on the surface of the virion (Hayes et al 1981, Lee et al 1981a). λ3 is thought to be the viral transcriptase (Dryna and Fields 1982). The functions of the remaining 3 core proteins λ1,μ1 and σ2, have not yet been determined, but because of their proximity to the viral dsRNA it is possible that they play a role in RNA synthesis (enzymatically or in a regulatory capacity) or in virus assembly and morphogenesis.

The virion outer shell consists of the polypeptides μ1c, μ2, σ3 and σ1. It is thought that μ1c and σ3 are associated in the virus particle since 80% of the unassembled forms of both these proteins in the cytoplasm of infected cells are complexed with each other (Huismans and Joklik 1976, Lee et al 1981a). It has been proposed that these two proteins may form capsomers in the ratio 1nμ1c:2nσ3, where n may be 2, since μ1c exists as a disulphide bonded dimer in virus particles (Smith et al 1969). Even though these two proteins are closely associated, they can also react independently, when virus particles are converted to sub-viral particles (see later) σ3 is removed totally, but μ1c loses a polypeptide with a molecular weight of about 4000 and

becomes converted to protein δ (Silverstein et al 1970, 1972, Chang and Zweerink 1971). μ1c has been shown to control the susceptibility of the Reovirus outer capsid shell to proteolytic digestion (Rubin and Fields 1980). σ3 has a strong affinity for dsRNA, an unexpected property for an outer shell protein suggesting that σ3 may play a role in virus assembly and/or morphogenesis.

σ1 is present in the virus at the level of only 24 molecules/virion (Joklik 1981). However, it is a very important protein, having several functions identified to date. σ1 is located close to the λ2 projections on the viral surface and is the Reovirus attachment protein (Lee et al 1981b). It is also the haemagglutinin (Weiner et al 1978), elicits the formation of neutralising antibody (Weiner and Fields 1977), is responsible for the development of delayed hypersensitivity (Weiner et al 1980) and for the generation of suppressor T-cells (Fontana and Weiner 1981) and cytolytic T-lymphocytes (Finberg et al 1978). This protein which also specifies Reovirus tissue tropism and segregates with virulence in Type 1 X Type 3 reassortants (Weiner et al 1977) is the most type specific of the Reovirus proteins, the polypeptide from the three Reovirus serotypes being antigenically very distinct.

The two non-structural proteins are known as μns and σns (Zweerink et al 1971). The functions of these two proteins are still unknown, and unlike non-structural proteins in other viruses they are produced in the infected cells in relatively large amounts. σns is interesting since it has a strong affinity for ssRNA, suggesting a function in morphogenesis,

possibly at the stage of assembly of the 10 plus-stranded RNAs for encapsidation (see later), or control of the translation of Reovirus mRNAs.

Some of the Reovirus proteins are well understood in terms of their location and function, while others remain largely uncharacterised. For example it is known that protein λ3 is involved in transcription, but the identity of the proteins involved in RNA synthesis and assembly of progeny virus particles is not known. The assembly of one copy of each of the 10 Reovirus genes is a very improbable event, and so it is thought that one or more of the above proteins must have a regulatory role.

D. REOVIRUS ENZYMIC ACTIVITIES

In 1968 Borsa and Graham, Shatkin and Sipe (1968a,b) and also Skehel and Joklik (1969) independently discovered an enzyme in Reovirus cores capable of transcribing ssRNA from a dsRNA template; the RNA polymerase or transcriptase. This is one of 5 enzymes encoded by and contained in Reovirus particles which are necessary for the production of functional mRNA molecules during infection. The other 4 are a nucleotide phosphohydrolase (Borsa et al 1970, Kapuler et al 1970) terminal guanyltransferase (Shatkin 1974, Furuichi et al 1975) (all Reovirus mRNAs are capped at the 5'end) and two methylases, one which methylates the cap-G, and one which methylates the ribose of the original 5'-terminal. Because these enzyme activities are

formed by an association of two or more proteins of the viral core. So far, with the exception of the viral transcriptase itself which has been tentatively identified as protein λ3 (Dryna and Fields 1982) the identity of these enzymes remains unknown. All are inactive in intact virions, and it is only when the outer protein shell is removed upon infection, that the enzymes become active.

E. RNA OF REOVIRUS

The viral genome consists of 10 segments of dsRNA, falling into three size classes; small, medium and large. From the work of McCrae and Joklik (1978) the coding assignments of all 10 species are known. In addition to the 10 dsRNA genes, virions also contain many small ssRNA oligonucleotides, accounting for about 25% of the total RNA in the particle (Bellamy and Joklik 1967, Shatkin and Sipe 1968a), these are located between the viral core and the outer shell (Bellamy et al 1972, Nichols et al 1972). As these oligonucleotides have "ppp" at their 5'ends they are thought not to be breakdown products of mRNA, but the result of abortive transcription. It is known that Reovirus transcriptase catalyses mRNA synthesis by repetitive initiation events, and that between 90 and 99% of these events results in transcripts of upto only 5 residues long, mainly pppGCU-OH and pppGCUA-OH (Yamakawa et al 1981). From studies using uv irradiation of viral cores, Henderson and Joklik (1978) report that transcriptional initiation is only followed by elongation if the template is functionally open along its entire length, and it has been shown by Harvey et

al (1981) by X-ray diffraction that RNA molecules are tightly packed within the core, adjacent RNA molecules being packed parallel to each other. It is therefore thought that in complete virus particles, the transcriptase can initiate RNA synthesis, but because the template is structurally unavailable, only short oligonucleotides can be produced. This is supported by the observation that it is not the transcriptase itself which is activated during infection, but rather the movement of the template segments relative to the catalytic sites (Yamakawa et al 1982).

F. ACTIVATION OF THE TRANSCRIPTIONAL ACTIVITIES

Proteolytic digestion of Reovirus particles releases the constraints on transcriptional elongation, due to a conformational change in the viral cores which allows the formation of non-covalent associations between protein and RNA (Powell et al 1984). Activation is therefore assumed to result from a conformational change in the genome such that movement of the RNA relative to the enzymic sites is possible and the RNA segments become functionally open along their entire length. The activation process has at least two separate steps (Borsa et al 1973,1974a,b). Firstly proteolytic digestion converts the virus particle into "intermediate" sub-viral particles (ISVPs) that have lost protein s3 and the COOH-terminal portion of protein u1c to yield protein d. ISVPs are resistant to further proteolytic digestion, but are still unable to elongate RNA. The second step, dependent upon a physiological concentration of Potassium ions

(such as that found in the cell cytoplasm) results in the formation Sub-Viral Particles (SVPs) which are capable of RNA elongation and are the particles which synthesise mRNA in infected cells (Borsa et al 1974a). This second step is thought to be controlled by a Reovirus endogenous protease, although there is no direct evidence for the existence of such an enzyme. In an infection, attachment of parental virions to the cell surface receptors (via the outer shell s1 protein) is followed by phagocytosis (Silverstein and Dales 1968), the phagocytic vacuoles fuse with lysosomes, where the proteolytic digestion takes place, and the resulting SVPs are then released into the cytoplasm where the active trancriptase is able to carry out mRNA synthesis. Full-length mRNA trancripts are extruded out of the viral cores via the l2 spikes into the cytoplasm (Gilles et al 1971), where they function at least initially in protein synthesis. It is postulated that the transcriptase catalytic sites are at the base of the spikes, facilitating this process.

G. REGULATION OF TRANSCRIPTION

In an in-vitro transcription system using Reovirus cores to synthesise mRNA, the level of synthesis of a gene is inversely proportional to its size. That is transcription rate is constant for all genes, and so more copies of the small genes are produced relative to the large ones, as they are initiated more often (Skehel and Joklik 1969).

The in-vivo situation is more complex, and transcriptional regulation clearly plays a role in Reovirus replication. Nonoyama et al (1974) found that the pattern of transcription varies during the infection cycle. Only four of the RNA segments are transcribed prior to 2 hours post-infection (PI), those being l1, m3, s3 and s4. By 2 hours PI between 5 and 7 of the segments are transcribed and by 4 hours PI all segments are transcribed, but at unequal frequencies. In permissive cells treated with cycloheximide, expansion of transcription to all 10 genes is prevented (Watanabe et al 1968) and the early pattern persists. A similar restriction is observed in untreated virus-cell combinations which are non-permissive for replication (Spandidos and Graham 1976), and SVP's taken from cycloheximide treated cells can synthesize all 10 mRNAs in vitro, but become restricted to l1 m3 s3 and s4 when reinfected into cycloheximide treated cells (Shatkin and LaFiandra 1972). It has been shown by Lau et al (1975) that these early transcripts are funtional mRNAs, they are translated immediatley following removal of the cycloheximide block into authentic viral polypeptides. Together these results suggest that a host protein may be involved in transcriptional regulation, but its nature and identity remain to be determined.

When all the Reovirus genome segments are being transcribed in infected cells the quantity of each transcript produced is still regulated. There are more small RNA's produced in vivo as well as in vitro, indicating that the small RNA's must be initiated at a higher frequency than the larger ones, since they are found in a 20 fold or more molar excess, while being only 25-30% of the length.

Although it has been demonstrated that Reovirus transcription is subject to control nothing is known about any mechanisms involved other than the suggestion above that host protein synthesis may influence the progression of an infection. It is likely one or more of the Reovirus proteins is involved in transcriptional control and again the core proteins are the most likely candidates for such a role. The unequal transcriptionn of the Reovirus genes supports the concept that the dsRNA genome is truly segmented, and is not linked in any way during transcription.

H. TRANSLATION OF REOVIRUS RNA

A mechanism for the regulation of transcription was proposed by Skup and Millward (1980a,b); during the course of infection the host cell translational apparatus appears to undergo a virus-induced switch from cap-dependence to cap-independence, which results in preferential translation of the uncapped mRNA produced by the progeny virions whose guanyltransferase and methylase enzymes are inactive. However this observation has not yet been confirmed and its importance is therefore unknown.

The rate of translation of the individual mRNAs is controlled (Zweerink and Joklik 1970), but the relative rates do not differ between early and late times post infection, and appear to be a function of the individual RNA molecules (Gaillard and Joklik 1985). Table 2 shows the relative frequencies of transcription and translation of the 10 Reovirus genes. It is likely that the signals for frequent or infrequent translation are contained in the sequences of the genes themselves. Joklik

TABLE 2: TRANSCRIPTION AND TRANSLATION FREQUENCIES OF THE 10 REOVIRUS GENES.

(Reproduced from Joklik 1981).

GENE	TRANSCRIPTION FREQUENCY	TRANSLATION FREQUENCY	TRANSLATION FREQUENCY / TRANSCRIPTION FREQUENCY
L1	0.05	0.03	0.6
L2	0.05	0.15	3
L3	0.05	0.1	2
M1	0.15	0.03	0.2
M2	0.3	1.0	3.3
M3	0.5	0.5	1
S1	0.5	0.05	0.1
S2	0.5	0.2	0.4
S3	1.0	0.3	0.3
S4	1.0	0.7	0.7

et al (personnal communication) have recently reported that sequences contained in the 5' untranslated regions of some mRNA molecules may determine efficiency of translation.

I. REOVIRUS MORPHOGENESIS

Reovirus assembly begins at the stage of ssRNA molecules (dsRNA is never found in free unencapsidated form in infected cells (Gomatos 1967)). Morgan and Zweerink (1975) observed early immature virus particles in cells infected with ts mutants of Reovirus which appeared to consist of several Reovirus proteins namely l1, l2, s2 and u1c and ssRNA molecules (Acs et al 1971). More recently Joklik and coworkers (Personnal communication) have looked for such assembly complexes at an earlier stage of infection (2 hours post-infection). They labelled RNA and protein during infections with both wild-type and ts-mutants (at permissive and non-permissive temperatures) and then immunoprecipitated assembly complexes using monoclonal antibodies directed against Reovirus RNA and proteins. They found that uns, u1c, s3 and sns play an important role in early morphogenesis. The two non-structural proteins uns and sns seemed to be of particular significance, and it appeared that after creating the initial assembly complexes, addition and exchange of Reovirus proteins occurs, resulting in the formation of core-like particles (Zweerink et al 1976) in which the transcriptase was still active. The ssRNA was then transcribed into dsRNA to form the progeny genomes, which could then be "secondarily transcribed" to produce more mRNA molecules. 95% of the viral RNA found in

infected cells is infact produced at this stage of replication (Morgan and Zweerink 1974. Sakuma and Watanabe 1972). Since it has proved difficult to isolate any of these assembly complexes from infected cells, it remains questionable exactly how many of the Reovirus transcripts are present in the particles; whether all 10 species are present is unknown. The observation by Zweerink (1974) that the Reovirus genes are copied into minus-strands sequentially suggests that they may be linked at this stage. The final stage of morphogenesis appears to be the inactivation of the viral transcriptase by the addition of protein σ3 (Astell et al 1972).

3. MODELS OF REPLICATION IN OTHER RNA VIRUSES

In order to achieve a productive infection, a virus has to produce the structural components of its virion by transcription and translation of its genome, and multiple copies of the genome itself. Since the work presented in this thesis is aimed towards a fuller understanding of how these two things are achieved in Rotaviruses, an appraisal of strategies employed in the other RNA viruses is appropriate. The Retroviruses, which replicate via a dsDNA intermediate will not be discussed.

There are three groups of viruses whose life cycle involves RNA as the only nucleic acid, in the Baltimore scheme of classification these are Class I (dsRNA), Class IV (ssRNA, mRNA sense) and Class V (ssRNA, minus sense).

CLASS III

This class of viruses comprises the Reoviridae which includes Rotavirus. As the replication of Reovirus RNA has already been discussed in Section 2 it will not be reconsidered here.

CLASS IV

Class IV can be further divided into two groups, differing in the way virus encoded proteins are produced. They have a single stranded unsegmented RNA genome, which is of the same sense as messenger RNA.

GROUP IVa consists of the Picornaviruses, including Foot-and-Mouth Disease Virus (FDMV) and Poliomyelitis (polio) virus, which is the best understood. Genomic RNA is polyadenylated at the 3' end, and is covalently attached to a small protein called Vpg at the 5' terminus. Because eukaryotic cell ribosomes are unable to begin translation at an internal initiation site, this virus has a problem in that only the 5' terminal sequence of its mRNA will be translated. To overcome this Poliovirus produces a polyprotein containing all the protein sequences encoded by the mRNA, and this is then cleaved to yield the final products. Cleavage is thought to be mediated by a host cell protease and a virus encoded enzyme (Kitamura et al 1981, Hanecak et al 1982). Poliovirus replication takes place in the cell cytoplasm and synthesis of the genome cannot commence until all the viral proteins have been produced and the RNA polymerase is active. It is thought that numerous minus sense strands are synthesized from the plus sense template with the viral protein Vpg donating a primer (of unknown structure) from which the nascent stranda are

elongated (Van Dyke et al 1982, Crawford and Baltimore 1983). A second amplification step then takes place where as many as eight plus sense strands are synthesized using the newly made minus strands as the template, in a replicative intermediate (Rueckert 1985). During the replication of poliovirus there is a switch in the function of the mRNA; early in infection it is used predominantly for protein production and later it is packaged into virions. It is thought that Vpg may be involved in this switch as it is found on mRNA which is packaged but not on that isolated from polysomes. However it does not prevent translation of the mRNA completely since the infecting RNA genomes possess it and are translated. No such protein has been found in the Rotavirus genome to account for any similar dichotomy of function (Section 1(ii)b).

Group IVb members also have a plus-sense RNA genome, their method of replication is similar to that of the picornaviruses. This group includes the Togaviruses which comprise the Alphaviruses (eg. Semliki Forest virus) and the Flaviviruses (eg. Yellow Fever virus). Two of the alphaviruses, SFV and Sindbis virus have been investigated in most detail. The genome of SFV and Sindbis virus is termed 49s RNA due to its sedimentation coefficient. Replication proceeds in the same way as in the picornaviruses, via a minus sense RNA molecule with amplification occurring at both stages. Viruses of this group produce two polyproteins; the first which is the translation product of the 49s RNA represents the 5' two-thirds of the genome and consists of four non-structural proteins. After the first three proteins there is a weak stop codon which, if read through adds the fourth protein which is followed by a strong stop-codon

(Collins et al 1982, Strauss and Strauss 1982, Strauss et al 1983). The second is the translation product of a 26s mRNA which is derived from the 3' third of the genome by internal initiation by an RNA dependant RNA polymerase. This is produced in much larger quantities than the 49s product, and consists of the viral structural proteins. Thus the virus is able to regulate the production of its proteins quantitively. How the production of the two RNAs is regulated and why some 49s is packaged, transcribed or translated is unknown (Schlesinger 1985).

CLASS V

Class V contains those viruses having a single -stranded minus sense RNA genome. The most fully understood member of the family is influenza virus which has a genome divided into 8 segments.

Following infection, influenza genomic RNA is released into the cell cytoplasm where it functions as template for the synthesis of mRNA by viral RNA dependent RNA polymerase. Since Influenza virus neither contains nor encodes the enzymes necessary for the addition of a cap at the 5' end of its mRNA, it uses the caps of cellular mRNAs to prime the polymerisation event. Cellular mRNA is cleaved between the cap and the first AUG and the new influenza mRNA retains the cap structure becomes polyadenylated and may then be translated. Plus sense copies of the viral genome which function as template for the synthesis of more genomic RNA (minus sense) do not become capped (or polyadenylated) in this fashion thus achieving an elegant system for differentiating two functionally distinct sets of mRNA; one for translation and the other for replication (Krug et al 1981).

As it is segmented, Influenza is able to regulate transcription of the genes individually in order to produce different levels of the viral proteins (there is no evidence as yet for translational control). All but two of the genes of Influenza A are monocistronic. Segments 7 and 8 are both bicistronic (as is gene 6 of Influenza B virus) the production of the second mRNA species is achieved by splicing which positions the 5' cap of the mRNA adjacent to the internal initiation codon.

The Rhabdoviruses and the Paramyxoviuses are also members of group V, but their genome is composed of a single piece of RNA. While genomic replication is principally the same as in Influenza virus, the method by which mono-cistronic mRNAs are produced differs. The products of Vesicular Stomatitis Virus (VSV) transcription in vivo are 5 mono-cistronic mRNAs, (capped and polyadenylated) and a leader sequence. The products of 3 of these mRNAs are needed for transcription of the genome. In order to account for the production of the 5 mono-cistronic mRNAs acceptable to the host-cell machinery two suggestions have been made; firstly, each gene may have its own promoter and polymerase site (Chanda and Banerjee 1981), secondly there may be only one polymerase entry site at the 3' end of the genome with polymerase molecules able to stop transcription at the end of a gene and re-initiate it at the beginning of the next one, or else synthesise one transcript which can be cleaved by a nuclease (Emerson 1982, Iverson and Rose 1982). As yet there is no convincing evidence for either of these possibilities (Emerson 1985).

Since the Rotavirus genome is similar to that of Reovirus it is probable, though by no means certain that replication will be carried out in the same way.

4. RATIONALE OF THIS WORK

As described in Section 2, Reovirus replication at the molecular level is understood in some detail, the roles of both transcriptional and tran slational control have been studied. The overall aim of the Rotavirus work at Warwick is the production of an anti-Rotavirus vaccine and this will be assisted by a more complete understanding of the events taking place in the infected cell. The aim of the work presented in this thesis was to characterise at the molecular level the replication of the Bovine (UKtc) strain of Rotavirus.

Due to its similarity with Reovirus, it has been assumed that Rotavirus RNA replication will proceed in the same fashion; fully non-conservatively (see Sections 1iii and 2) and there is now some evidence to indicate that this is probably the case (Patton 1986). Reovirus replication was therefore used as a model for the methodology described in this thesis. Reovirus transcription was studied by Nonoyama et al (1974) by labelling the RNA synthesised during an infection of tissue culture cells with 3-H uridine, and then annealing this with Carbon-14 labelled genomic dsRNA. The hybrids were then fractionated by PAGE and relative production of the 10 genome segments was measured throughout the infection by counting radioactivity in gel slices.

However this method does not discriminate between plus and minus sense strands of the genome which for us was particularly important, and because of the migration pattern of Rotavirus RNA (some genes such as 2 and 3 require prolonged electrophoresis for complete resolution) this method would have been difficult to apply. We wished to molecularly dissect the production of Rotavirus RNA; to analyse both strands of each gene in turn in order to build up a complete picture of Rotavirus transcription and replication. Using cloned cDNA copies of all 11 Rotavirus genes which were already available in the laboratory (McCrae and McCorquodale 1982b, and personal communication), it was possible to make high specific activity and strand specific probes, and use these to develope a novel assay for the accumulation of RNA in Rotavirus infected (tissue culture) cells. To determine the relative importance of transcriptional and translational control in Rotavirus replication it was also necessary to examine the production of proteins, and as the gene coding assignments for the whole Rotavirus genome is known (Figure 2, McCrae and McCorquodale 1982b) it was possible to compare mRNA production to protein production for each gene.

For the production of these strand-specific RNA probes we chose to use the Gemini vector transcription system which allows the synthesis of a copy of either strand of a clone, free from vector sequences.

a. BACKGROUND TO THE TRANSCRIPTION SYSTEM

The Gemini-vector based transcription system was chosen for the generation of single-stranded RNA probes to the 11 Rotavirus genes. This system is based on two RNA polymerases, SP6 and T7.

b. SP6 POLYMERASE

SP6 is a small virulent bacteriophage which grows in Salmonella typhimurium LT2. It was first isolated and characterised in 1982 by Butler and Chamberlin, who were able to syntheise SP6 RNA, using the SP6 containing DNA as template. The purified SP6 would only respond to the homologous promotor sequence; it was inactive when put into a system containing other bacteriophage DNAs including T7 and T3. SP6 polymerase proved easy to purify and was very stable, and so it could be obtained in the quantities and purity necessary for physical and chemical studies of structure and function of a small highly specific RNA polymerase. (Butler and Chamberlin 1982).

In the 2 or 3 years which followed, unprocessed RNAs synthesized in vitro by transcription from an SP6 promotor were sucessfully used to study RNA splicing (Kruger et al 1982, Green et al 1983, Hernandez and Keller 1983, Padgett et al 1983, Krainer et al 1984) tRNA maturation (Guerrier-Takada and Altman 1984) and 3' end formation (Manley 1983, Birchmeier et al 1984, Kreig and Melton 1984). In many cases single stranded RNA probes were found to be more convienient to prepare and more sensitive than dsRNA or Nick-translated DNA probes, and as a consequence

have improved the detection sensitivities of blot hybridisation procedures, and in-situ hybridisations (Brahic and Haase 1978, Diaz et al 1981, Cox et al 1984).

In order to facilitate the transcription of large amounts of specific RNA and the generation of high specific activity probes, one needs a transcription system where the initiation and termination of transcription is well defined and at precise points on the DNA template. In addition, in order to make the production of such transcripts more practical, an in-vitro system is desirable, hence eliminating the need for the production of cell extracts. In 1982, two groups of workers described an in-vitro system in which the SP6 polymerase was used to transcribe DNA situated downstream of the SP6 promotor sequence. Transcripts produced in this system were specific to the SP6 promotor, and spurious transcripts (for example from the other DNA strand) were rarely seen. The transcription conditions for this system consisted simply of salt buffers, template DNA, RNA substrates (ribonucleotides) and the polymerase itself (Butler and Chamberlin 1982, Kassavetis et al 1982). Based on these observations Melton et al (1984) constructed a new set of cloning vectors which contained an SP6 RNA polymerase promotor upstream of a polylinker sequence, making it convienient to clone any DNA sequence downstream, and make RNA transcripts. These vectors are known as the pSP64 and pSP65 transcription vectors, they differ in the orientation of the polylinker with respect to the SP6 promotor.

c. T7 RNA polymerase

The Escherichia coli bacteriophage T7 is morphologically very similar to the SP6 bacteriophage described above. Like SP6, T7 RNA polymerase has a stringent specificity for its own promotors, of which there are 7 in the T7 DNA. However few if any of these promotor sequences are found in unrelated DNAs. T7 polymerase made during T7 infection of E.coli was purified and shown to direct the transcription of genes cloned in plasmids downstream of the promotor sequence (Campbell et al 1981, McAllister et al 1981, Studier and Rosenberg 1981). The T7 coding sequence was then cloned into a suitable expression vector, pBR322 and put under the control of the inducible lacUV5 promotor, the final construct called pAR1219 gave rise to large amounts of T7 RNA polymerase which could then be purified (Davanloo et al 1984).

d. THE GEMINI VECTORS

The Gemini vector system was constructed at Promega Biotec. These vectors of which there are four, denoted pGEM 1,2,3 and 4, contain both the SP6 and T7 promotors described above flanking a pUC18 derived multiple cloning region. pGEM 1 and pGEM 2 form a pair differing only in the orientation of the polylinker with respect to the RNA polymerase promotors. pGEM 3 and 4, which form a similar pair contain an Sph1 and a Kpn1 site in the polylinker in addition to the 11 already present in pGEM 1 and 2. The structure of the polylinker region of pGEM1 is shown in Figure 3. By inserting a cloned DNA sequence into this polylinker region it is straightforward to make RNA copies of both strands of the DNA from a single vector simply by selecting the appropriate

FIGURE 3: THE POLYLINKER REGION OF pGEM1.

The cloning vectors used for the work described are the Gemini transcription vectors, which possess SP6 and T7 RNA polymerase promotors flanking a polylinker region composed of eleven unique restriction enzyme sites. The order of the sites with respect to the promotors is reversed in pGEM2 and the arrows indicate the direction of transcription initiated from each promotor.

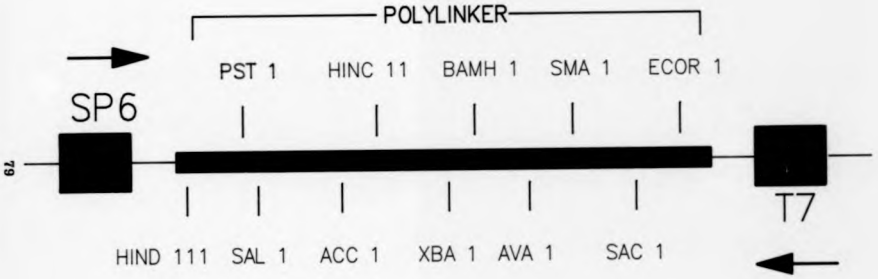


FIGURE 3: THE POLYLINKER REGION OF PGEM1

transcriptase. This corresponds to the plus or minus sense strand of the Rotavirus genome. In order to make transcripts a defined length, the DNA template is linearised prior to transcription at a restriction enzyme site distal to the promotor being used, transcripts are therefore run-off products. Using this system it has been possible to generate strand specific probes for each of the 11 Rotavirus genes. We wished to use these probes to quantitiate the transcription and replication of the Rotavirus genome throughout infection. We chose to use an adaptation of S1 nuclease mapping, "RNAase mapping" as we believed that this could generate convenient numerical and quantitative results, and would be more rapid and accurate than traditional methods such as Dot-Blotting (Pedley and McCrae 1984).

e. S1 NUCLEASE MAPPING

S1 nuclease degrades single-stranded DNA and RNA, but at low temperature and high ionic strength does not digest double-stranded nucleic acids. S1 nuclease has been used extensively to study splicing in eukaryotic mRNA (Berk and Sharp 1977). A cloned DNA probe is hybridised to the mRNA and the hybrid is treated with S1 nuclease which degrades unhybridised segments of the DNA probe to leave discrete DNA fragments the size of which is equal to the length of the nucleotide sequence over which there is perfect homology between the RNA and DNA. These double-stranded fragments are then analysed on agarose gels,

and have been used to define splice points in the mRNA which result in the production of two or more hybrid fragments from the one DNA sequence (Williams and Mason 1985).

RNAase mapping is analogous to S1 nuclease mapping. In this case a high specific activity RNA transcript is hybridised to the test RNA and the resulting hybrids are treated with single-strand specific RNAases, which digest away any unhybridised RNA (Zinn et al 1983). The RNAase resistant (ds) fraction can then be collected and analysed. By including suitable standards in the assay, the exact amount of homologous RNA present in the sample can be calculated. Using this method it has been possible to measure molar amounts of both strands of the 11 Rotavirus genes produced during one-step growth in a tissue culture system and relate this to the levels of protein products produced. The development of the Solution hybridisation assay is described in detail in Chapters 3 and 4 (Results).

MATERIALS AND METHODS.

MATERIALS AND METHODS

MATERIALS

All materials not listed were obtained as Analar grade from BDH Chemicals Limited, Poole, Dorset.

AGAR AIDS, Bishops Stortford, Herts: Electron Microscopy Grids.

AMERSHAM INTERNATIONAL LTD, Bucks: T4 Polynucleotide kinase, all radioactive isotopes.

BOEHRINGER CORPORATION LIMITED, Lewes: T7 RNA polymerase, SP6 RNA polymerase, RNAsin (from human placenta), Ribonuclease A, Proteinase K, Restriction Endonucleases (AccI, AluI, BamH1, BstN1, ECoR1, HinCII, HinDIII, HinfI, HpaII, PstI, SalI)

BECKMAN INSTRUMENTS LIMITED Fullerton, California: Scintillation fluid, EP.

BETHESDA RESEARCH LABORATORIES/GIBCO LIMITED, Scotland: T4 Kinase, DNA polymerase I, Klenow Fragment of DNA polI, Bovine Serum Albumin (Nuclease free), T4 RNA ligase, Phage Lambda DNA, Tissue culture dishes.

BIORAD, Richmond California: Bis-Acrylamide, Ammonium persulphate, Sodium lauryl sulphate, TEMED.

COOPER BIOMEDICALS (Agent; Worthington, Bury St Edmonds, Suffolk) Deoxyribonuclease I(RNase free).

EASTMAN KODAK COMPANY, New York, USA: X-Omat film, LX 24 X-ray developer, FX 40 X-ray fixer.

FLOW LABORATORIES LIMITED,Scotland: Fetal Calf Serum, BSC-1 cells, GMEM, Gentamycin Sulphate.

3MM LIMITED, St Paul, Minnisota, USA: Tape for taping up gel plates for polyacrylamide gels.

MAY AND BAKER LIMITED,Dagenham: Trichloroacetic acid, Sodium Citrate.

MILES SCIENTIFIC BIOCHEMICALS, Slough: Low melting point agarose: Seaplaque for Formaldehyde gels, SeaKem LE for Tissue Culture use.

NORTHUMBRIA BIOLOGICALS LIMITED, Northumberland: Fetal Calf Serum, Tissue culture dishes.

PALL ULTRAFINE FILTRATION CORPORATION, New York:Biodyne A Nylon transfer Membrane.

PHARMACIA BIOTECHNOLOGY, Uppsala, Sweden: Sephadex G50, Mung Bean Nuclease.

PROMEGA, through P&S BIOCHEMICALS LIMITED, Liverpool: Gemini Vector DNA pGEM1 and pGEM2.

POLAROID (UK) LIMITED, St Albans Herts: Type 55 Land film (positive/negative).

SIGMA CHEMICAL COMPANY LIMITED, Poole, Dorset: Ribonuclease T1, Dithiothreitol, tRNA, Salmon-Sperm DNA, Polyvinylpyrollidone, Ficoll, Bovine Serum Albumin Fraction V, Trypsin,type III.

WHATMANN INTERNATIONAL LIMITED, Maidstone, Kent: Hardened Ashless Paper, 2.5cm Filter paper No3, 2.5cm G/FC Glass microfibre filter.

METHODS

1. VIRUS GROWTH AND PURIFICATION

i. GROWTH

BSC-1 cells were maintained in GMEM+NEAA with 5% fetal calf serum and 50ug/ml Gentamycin sulphate. The Compton UK tissue culture adapted bovine Rotavirus was originally obtained from Dr M. Thouless. Virus stocks were grown by infecting confluent roller bottles of BSC-1 cells at a multiplicity of infection of 0.1PFU/cell. After absorption for 1 hour at 37°C, cells were overlaid with GMEM + NEAA containing Trypsin at a final concentration of 10ug/ml. Virus growth was allowed to proceed for 3 days at 37°c after which time all cells had become detached from the glass. Cells were counted using a haemocytometer.

ii. PURIFICATION

Purified virus was obtained from 40 X 650cm roller bottles grown as described above. Virus was purified using a modification of the procedure for Reovirus (Smith et al 1969). Cells and virus were concentrated by centrifugation at 100,000g and then cells disrupted by sonication, followed by homogenisation with one-quarter total volume of Arcton (trichlorofluorethane). Phases were separated by low-speed centrifugation and the aqueous phase decanted and reserved. The Arcton phase was then re-extracted twice with 25mls of Resuspension buffer (50mM Tris pH8.0, 10mM NaCl, 1.5mM β-mercaptoethanol, 3mM $CaCl_2$). All

aqueous phases were then pooled and "back-extracted" with 50mls of Arcton. Virus was concentrated by centrifugation at 100,000g for 1 hour at 4°C, the pellet resuspended in resuspension buffer and loaded onto a pre-formed $CsCl_2$ gradient of p1.2-1.4 which was centrifuged at 100,000g and 4°C for 2 hours. The p1.36 band was collected by side puncture, concentrated by centrifugation and resuspended in 50mM Tris pH8.0.

iii. EXTRACTION OF GENOMIC RNA FROM PURIFIED VIRUS

The viral suspension (1mg/ml) was made 1% with respect to SDS and extracted 3 times with water saturated phenol. Residual phenol was removed by extracting 4 times with diethyl ether and RNA was collected by ethanol precipitation in the presence of 0.15M Lithium Chloride. Concentration of the RNA was calculated on the basis of optical density at 260nm.

2. PREPARATION OF INFECTED CELL RNA

18 confluent roller bottles of BSC-1 cells were pre-labelled with ^{3}H-Uridine (2.5uCi/ml) for 16 hours prior to infection with UKtc Rotavirus at an MOI of >10 pfu/ml. 2 bottles were mock-infected. After absorption at 37°C for 1 hour cells were overlaid with GMEM+1% FCS. 2 Bottles of cells were harvested at hourly intervals for 8 hours and cells collected by trypsinization (0.05% trypsin) and low speed centrifugation. Pellets were resuspended in 10mls 50mM Tris pH8.0 and stored at -20°C, after withdrawal of 0.2ml which was stored at 4°C for

titration of infectivity on monolayers of BSC-1 cells (McCrae and Faulkner-Valle 1981). RNA was extracted by sonication of the cell preparation followed by 10 strokes with a Dounce Homogensiser. SDS was added to a final concentration of 1% and the suspension extracted once with phenol equilibrated with 0.1M Acetate buffer pH5.0 at 60°c and twice with water saturated phenol at 37°c. The RNA was precipitated with ethanol and the pellet was resuspended in 1500ul of formamide hybridisation buffer (see section 5ii) and balanced with respect to TCA precipitable tritium content by adjusting the volume.

3. IDENTIFICATION OF c-DNA AND SUB-CLONES

c-DNA clones specific to all eleven genes had already been subcloned into pAT153 (McCrae and McCorquodale 1982 and personal communication). Only three of these had to be identified from a collection of potential clones at the commencement of this work. c-DNA clones specific to genes 1,3 and 4 and the genetic origin of the sub-clones generated was identified by hybridisation of radioactively labelled c-DNA to fractionated Rotavirus genomic RNA immobilised by the Trans-blotting procedure.

i. FRACTIONATION OF ROTAVIRUS ds-RNA

The Rotavirus genome was fractionated by electrophoresis on 6% polyacrylamide gels using the Laemmli buffer system (Laemmli 1970). For fractionation of species 10 and 11 a 20cm gel and electrophoresis for 16 hours at 20mA was used.

Complete resolution of genes 2 to 4 and 7 to 9 required the use of 40 cm gels with electrophoresis for 52 hours at 20mA and resulted in the loss of genes 10 and 11 from the gel. Samples were mixed with one fifth volume of "cracking buffer" (10% SDS, 25% BME, 50% glycerol, 1X Laemmli stacking gel buffer and bromophenol blue) and heated to 70°c for 2 minutes prior to loading the gel.

ii. IMMOBILISATION OF THE RNA

Fractionated RNA was immobilised onto diazophenylthioether (DPT) paper according to the procedure described by Street et al (1982) and after Alwine et al (1979) and Stellwag and Dahlberg (1980) using a Biorad transblotting apparatus.

iii. LABELLING c-DNA CLONES

c-DNA was labelled with alpha-^{32}P-dGTP by nick translation; 100ng of plasmid DNA was incubated at 15° c for 3 hours with 1mM each of three unlabelled d-NTP's, 3ng/ml DNase 1, 20-30uCi alpha-^{32}P-dGTP and 4 units of DNA polymerase 1 in 0.5m Tris pH 9.0,50mM MgCl2, and 0.1M βME. The reaction was passed over a 5ml Sephadex G-50 column equilibrated with Column buffer (50mM Tris pH8.0, 500mM NaCl and 0.1% SDS) and the void volume peak collected, pooled and precipitated with ethanol.

iv. HYBRIDISATION TO IMMOBILISED GENOMIC RNA

DPT paper bearing Rotavirus RNA was cut into one-lane strips and sealed into individual plastic bags containing approximately 5mls of hybridisation buffer (5X Denhardt's solution (0.1% each polyvinylpyrollidone, Ficoll and BSA Fraction V in 2X SSC) 5X SSC (20X SSC stock solution is 1.5M Sodium chloride and 0.15M Sodium Citrate) 50mM Sodium phosphate buffer pH6.5, 0.1% SDS 250ug/ml Sheared salmon-sperm DNA (Maniatis 1982) and 50% Deionised formamide pH6.5), and incubated at 42° c for at least 2 hours. The buffer was then replaced with 2.5 mls of fresh buffer containing 10% w/v glycine together with the labelled DNA which had been denatured by boiling (10 minutes) and quench-cooling (in a dry ice/ethanol bath). Incubation was continued for at least 16 hours at 42° c (PALL corp.1985). Following hybridisation filters were washed 4 times for 5 minutes in (0.1% SDS and 2 X SSC) at room temperature and twice for 15 minutes in (0.1% SDS and 0.01 X SSC)at 50° c, before drying and autoradiography.

4. SUBCLONING c-DNA FRAGMENTS FROM PAT153 INTO pGEM1 AND pGEM2.

1. PREPARATION OF DNA

The c-DNA inserts were excised from pAT153 using appropriate restriction enzymes and conditions as described by Maniatis et al (1982) with the addition of 100ug/ml BSA to all restriction enzyme buffers. The required DNA fragment was isolated by agarose gel electrophoresis followed by electroelution (McDonnell et al 1977). pGEM1 and pGEM2 DNA was similarly digested and treated with Calf Intestinal Phosphatase to remove 5'

terminal phosphate residues (to minimise self-ligation of the plasmid). Insert and plasmid DNA were extracted twice with water saturated phenol, four times with ether and collected by ethanol precipitation.

ii. LIGATION AND TRANSFORMATION

c-DNA fragments were ligated with phosphatase treated plasmid DNA using a molar excess of insert to plasmid of 10:1. Ligation was allowed to proceed at 15° c overnight using 100ng of plasmid DNA, 2 units of DNA ligase and 1 unit of RNA ligase in ligation buffer (Maniatis et al 1982).

Ligated DNA samples were transformed into competant E.coli cells (strain MC1061). Competant cells were generated by diluting an overnight culture 1 in 100 in L-Broth (10gm Bactotryptone, 5gm Yeast extract, 5gm NaCl per litre) and incubating with agitation until the OD590 reached 0.4-0.6. Cells were washed in a half volume of 0.1M $MgCl_2$ by low-speed centrifugation, and resuspended in a twentieth volume of pre-chilled 0.1M $CaCl_2$. Cells were incubated on ice for 30 minutes after which time 200ul was added to each ligation reaction which had been adjusted to 100ul and 0.1M $CaCl_2$. Incubation on ice was continued for 30 minutes, and cells were then heat-shocked at 42° c for 2 minutes followed by further 30 minutes on ice. Aliquots were spread onto 1.5% L-agar containing 100ug/ml ampicillin. Since MC1061 is ampicillin sensitive and the Gemini vectors bear the ampicillin resistance gene any colonies growing on the plates after 16 hours incubation (37° c) contain the plasmid .

iii. SCREENING FOR RECOMBINANT COLONIES

At this stage no distinction can be made between colonies containing re-ligated plasmid and colonies containing plasmid plus c-DNA. Colonies were screened initially by mini-plasmid preparations as described by Birboim and Doly (1979). Plasmid DNA was digested with the restriction enzyme used for the insertion and analysed by agarose gel electophoresis alongside DNA size markers prepared by digestion of plasmid pBR322 DNA with the enzymes AluI (for small <800bp fragments) or Lambda DNA digested with ECoR1 and HinD111 and pBR322 digested with AvaI1 and BstN1 for the larger fragments. Normally several colonies containing c-DNA could be identified by this method. However in one case it was necessary to screen larger numbers of potential recombinants (400) since the frequency of insertion appeared to be very low. In this case the colony hybridisation method of Grunstein and Hogness (1975) was used.

iv. LARGE SCALE PLASMID PREPARATION

To prepare plasmid stocks of recombinant colonies 1 litre cultures were grown in L-broth until the OD590 reached 0.6, chloramphenicol was added to a final concentration of 150ug/ml and incubation continued at 37°c overnight to amplify the plasmid. Cells were then pelletted and re-suspended in 8.4ml 25% sucrose in 50mM Tris pH8.0 on ice. 1.4mls of lysozyme (10mg/ml) was added followed by incubation on ice for 5 minutes and the

addition of 4.6ml 0.25M EDTA. After a further 10 minutes 9.6ml Triton lysis mix (2% Triton, 0.0625M EDTA, 50mM Tris pH8.0) was added. Chromosomal DNA was pelleted by centrifugation (1 hour at 23,000g) and removed, the remaining supernatant was adjusted to 25ml with 50mM Tris and 25gm solid Caesium Chloride added. Ethidium bromide was added to 250ug/ml and the preparation was centrifuged for 1 hour at 23° c and 100,000g to remove solid matter, the cleared solution was transferred to a 38ml "Quickseal" tube and centrifuged in a Beckman Vti50 Titanium rotor at 45,000 RPM (180,000g) at 23° c for 16 hours in a Beckman L8 centrifuge. The plasmid DNA band could normally be seen by eye and was collected by side puncture. Ethidium bromide was removed by 3 extractions with CsCl saturated isopropanol and CsCl was removed by dialysis with 3 changes of 10mM Tris pH8.0, 1mM EDTA. DNA was precipitated with ethanol, resuspended and extracted 3 times with water saturated phenol. After a further precipitation step the OD260 of the plasmid solution was used to calculate its concentration.

5. IN VITRO TRANSCRIPTION

i. LINEARISATION OF PLASMID TEMPLATE

In order to obtain transcripts of a defined length from the DNA template it was neccessary to linearise the plasmid by cutting at a restriction enzyme site in the polylinker distal to the RNA polymerase promotor being used. Polymerisation then resulted in the synthesis of "run-off" transcripts. Most clones

were digested with Sal1 and HinDIII (genes 5-11) while for others Pst1, HinCII and BamH1 were used (see Table 4). 50ug of each plasmid was digested overnight with 5 units of enzyme using conditions outlined by Maniatis et al (1982). Complete linearization was confirmed by agarose gel electrophoresis and DNA was extracted with phenol and resuspended in water to a concentration of 1mg/ml.

ii. IN VITRO TRANSCRIPTION

The final conditions used for transcription are shown; At room temperature and in the order shown reagents were added together before incubation at 37°c for 60 minutes:

Synthesis of high specific activity transcript

Reagent	Concentration of stock	Volume taken	Final Concentration
Autoclaved distilled water		5ul	
Plasmid DNA	1mg/ml	2ul	2ug total
rUTP,rATP,rGTP	5mM each	2ul	500uM each
RNasin	20units/ul	1ul	1unit/ul
BSA	5mg/ml	.5ul	.125ug/ul
DTT	100mM	2ul	10mM
Transcription Buffer	5 X	4ul	1 X
32-P rCTP	800 Ci/mMol	2.5ul	50uCi
RNA polymerase	10units/ul	1ul	10 units

Synthesis of "unlabelled" transcript

To enable calculation of concentration following synthesis of the transcript RNA, a small amount of isotope is included in this reaction. 10uCi ^{3}-H rUTP or rATP (51 Ci/mMol) was vacuum dried to remove ethanol and resuspended in 25ul distilled water. The other reagents were then added:

Reagent	Concentration of stock	Volume taken	Final Concentration
Autoclaved distilled water		5ul	
Plasmid DNA	1mg/ml	2ul	2ug total
Four rNTP's	10mM each	2.5ul	500uM each
RNasin	20units/ul	2.5ul	1unit/ul
DTT	100mM	5ul	10mM
Transcription buffer	5 X	10ul	1 X
RNA polymerase	10units/ul	2ul	20units

5 X TRANSCRIPTION BUFFER: 200mM Tris pH7.5(at 37° C) 30mM MgCl2, 10mM Spermidine, 50mM NaCl)

Following incubation the DNA template was removed by digestion with 2 units of RNase-free DNase (37°c for 15 minutes). The reaction volume was then increased to 200ul with water and extracted twice with phenol and precipitated under ethanol using 0.7m Ammonium Acetate, and resuspended either in water or in 100ul of Formamide hybridisation buffer (1 X PIPES, 80% Formamide) by the sequential addition of 10ul water , 10ul 10X PIPES buffer (400mM PIPES pH6.7, 4M NaCl, and 10mM EDTA) and 80ul of deionised formamide pH 6.7 prepared using Amberlite monobed resin. (Okayama and Berg 1982)

iii. ANALYSIS OF TRANSCRIPTS

A. BY GEL ELECTROPHORESIS

Transcripts were analysed on denaturing formaldehyde agarose gels to confirm that complete copies of both strands of the DNA were being made. 1.5% gels were cast in MOPS buffer (0.02M MOPS, 5mM Sodium Acetate, 1mM EDTA, adjusted to pH7 with NaOH) and 6% Formaldehyde. Samples were balanced with respect to TCA precipitable radioactivity and aliquots adjusted to 5ul with water. 15ul of denaturing solution (500ul deionised formamide, 100ul 10X MOPS buffer, and 150ul formaldehyde) was added to each aliquot, and samples were heated to 60° c for 15 minutes followed by quench cooling on ice. Samples were transferred to room temperature and 2ul loading mix (50% Glycerol, 0.2% Bromophenol blue, 0.2% Xylene Cyanol FF) was added. Samples were loaded onto

the gel immediately and electrophoresis in 1 X MOPS buffer continued for 4-8 hours at room temperature. At the end of electrophoresis gels were dried and exposed for autoradiography.

B. BY DOT-BLOT

Samples of Rotavirus genomic and mRNA were resuspended in 50-100ul of water and boiled for 10 minutes to denature followed by quench-cooling on ice. An equal volume of pre-cooled 20 X SSC was added and samples loaded onto Nylon Membrane which had been equilibrated with 20 X SSC, by means of a Biorad "BIO-DOT" manifold. The loaded membrane was then baked in vacuo for 2 hours at 80° c before pre-hybridisation and hybridisation as described in Section 3iv.

6. SOLUTION HYBRIDISATION

i. PREPARATION OF STANDARD RNA STOCKS

A. mRNA

Rotavirus mRNA was synthesized by virions following activation of the virion associated RNA dependent RNA polymerase (Cohen 1977) using the conditions described by Mason et al (1980).

B. "Unlabelled" transcript

"Cold" transcript stocks of each strand of the Rotavirus specific cDNA subclones were prepared as described in section 5ii. Precipitation of transcripts with Ammonium Acetate

(in preference to Lithium Chloride) reduces the quantity of mononucleotides co-precipitated. The ^{3}-H rNTP was included in the transcription reaction to facilitate calculation of RNA concentration based on incorporation and specific activity of the tritiated triphosphate.

ii. THE SOLUTION HYBRIDISATION

High specific activity RNA (probe) was resuspended in formamide hybridisation buffer to contain 50,000 TCA precipitable CPM per ul. Test RNA was prepared in 20ul of the same buffer, and 10ul of probe added to each sample. The samples were then heated to 85° c for 10 minutes and hybridisation allowed to proceed for 16 hours at 42 c.

Transcript-viral RNA hybrids were collected by first removing unhybridised RNA by treatment with RNase's A and T1 (using low-salt conditions in which they are single-strand specific): 150ul RNAse buffer (10mM Tris-HCl pH7.5, 5mM EDTA, 300mM NaCl) was added to the hybridisation reaction, which was transfered to and mixed with 150ul RNase buffer containing 2ug/ml RNase T1 and 40ug/ml RNase A. Digestion was allowed to proceed for 2 hours at 30° c and was terminated by the addition of 100ug Proteinase K, 10ug tRNA and 20ul 10% SDS with further incubation at 37°c for 15 minutes.

RNase resistant RNA was collected by TCA precipitation in solution. To each sample was added 45ul of 100% TCA, 45ul 200mM Sodium Pyrophosphate and 450ul of 10% TCA contailng 20mM Sodium pyrophosphate. The precipitate was allowed

to form for 10 minutes on ice, and was collected by filtration onto 2.5cm Whatman glass fibre discs under vacuum by means of a Millipore filter manifold. Filters were washed once with 10% TCA containing 20mM Sodium pyrophosphate and twice with 100% ethanol, and dried by baking at 60°c for 1 hour. Counting was carried out using the LKB 1219 Rack-beta scintillation counter, and Beckman EP scintillation fluid. The scintillation counter was set up such that the two isotopes being used were counted in separate channels without spillover.

iii. ANALYSIS OF dsRNA HYBRIDS FORMED

Examination and size determination of the dsRNA hybrids formed was carried out by phenol extraction and precipitation of hybrids following the addition of proteinase k. Hybrids were loaded onto 5% polyacrylamide gels (cast and run in 1 X Loenings buffer (36mM Tris-HCl, 30mM NaH_2PO_4, 1mM EDTA)) and electrophoresis carried out alongside labelled size markers. Time of electrophoresis varied between 6 hours at 50mA for the smallest hybrid, and 48 hours at 30mA for the largest (40cm gel). Gels were dried prior to autoradiography.

7. ANALYSIS OF PROTEIN SYNTHESIS

Protein synthesis was studied by pulse-chase analysis of infected cells with ^{35}S labelled Methionine (800Ci/mMol). Cells were incubated in methionine-free medium for 16 hours prior to infection in order to minimise isotopic

dilution. Infection with an MOI of 10PFU/cell, was allowed to proceed for 6.5 hours before labelling. Where a chase sample was required medium containing 100 X the normal concentration of cold methionine was added to cells following the removal of the isotope. Samples were balanced with respect to precipitable ^{35}-S content and run on 5-11% gradient polyacrylamide gels using the Laemmli discontinuous buffer system (Laemmli 1970). Electrophoresis was continued for 6 hours at 30mA and gels were treated with PPO (2,5 Diphenyloxazole) to allow fluorography and dried. Where required individual protein bands were excised from the gel using exposed film as the template and counted individually. When cycloheximide was used this was added 2 hours prior to infection at 20ug/ml and maintained until indicated.

8. PHOTOGRAPHIC RECORDS OF GELS BEARING DNA AND RNA

Following electrophoresis gels were stained with Ethidium Bromide (5ug/ml for 15 minutes) and photographic records made using Polaroid type 55 (positive/negative) Land film.

9. MEASUREMENT OF INCORPORATED RADIOACTIVITY

Radioactive isotope incorporated into macromolecules (eg RNA transcript, DNA fragments or protein) was measured by precipitation with Trichloroacetic Acid (TCA). Aliquots were applied to Whatman 3MM filter paper and treated as follows; For measurement of ^{32}-P incorporated into nucleic acid, filters were washed once for 10 minutes and once for 5 minutes in

cold (on ice) 10% TCA plus 20mM Sodium Pyrophosphate. For measurement of ^{35}S incorporated into proteins the filters were boiled for 10 minutes in 10% TCA containing 20% Cas-amino acids. All filters were then washed twice in 100% ethanol, and twice in diethyl ether before drying and counting in Beckman EP scintillant.

10. **AUTORADIOGRAPHY**

Gels or filters bearing radioactive isotopes were exposed to X-OMAT-S film, for times varying between several hours to several weeks depending on the amount and type of radioactivity present. Gels bearing ^{35}-S were exposed at -70° c, those bearing ^{32}-P were exposed at room temperature or at -70° c using a lightening-plus intensifying screen (DuPont) to shorten exposure time. Where possible gels bearing ^{32}-P were exposed without a screen to achieve a better resolution.

11. **PREPARATION OF dsDNA SIZE MARKERS AND LABELLING ROTAVIRUS RNA.**

Size markers used in analysis of sub-clones, transcripts and hybrid RNA were restriction enzyme digests of Lambda or plasmid pBR322 DNA. Typically 10ug of DNA was digested with 2 units each of the enzymes being used; Lambda DNA was cut with ECoR1 and HinDIII, and pBR322 with HpaII, AvaII or BstNI. Where labelled markers were required these were obtained by incubating the cut Lambda DNA and the HpaII digest of pBR322 with 10uCi of alpha-^{32}-P dGTP and the Klenow fragment of DNA polymerase

I (Sanger 1977). Labelled DNA was then collected by passing the reaction over a 5ml Sephadex G-50 Column, and precipitation of the void volume peak.

Rotavirus messenger and genomic RNA were labelled using with T4 polynucleotide kinase and gamma labelled ^{32}-P ATP: The RNA was digested with 50mM NaHCO (90° C for 4 minutes) to expose numerous 5'-OH termini accessible to the labelling procedure. The RNA was then precipitated and the pellet resuspended in 6ul of water to which 35ul of buffer A [20mM Tris pH9.5, 1mM Spermidine, 0.1mM EDTA], 5ul of buffer B [500mM pH 9.5, 100mM MgCl , 50 mM DTT, 50% glycerol] 25uCi gamma-^{32}P ATP and 4 units T4 polynucleotide kinase were added. The reaction was allowed to proceed for 15 minutes at 37°C, after which time labelled RNA was collected using a Sephadex G-50 column.

12. DETERMINATION OF PARTICLE:PFU RATIO.

The particle:PFU ratio of the 8.5 hour sample was determined by mixing an equal volume of infected sample with an appropriate dilution of a solution of latex beads of known concentration. The ratio of virus particles to latex beads was counted by electron microscopy (using uranyl acetate for staining), and used to calculate the number of virus particles per ml which could then be compared to the number of infectious units (PFU) per ml.

RESULTS: CHAPTER 1.

CHAPTER 1

SUBCLONING AND CHARACTERISATION OF ROTAVIRUS c-DNA CLONES

INTRODUCTION

As outlined in Section 4 (Introduction) the aim of the work presented in this thesis was to characterise the replication of Rotavirus (in tissue culture) at the molecular level. In order to molecularly dissect Rotavirus transcription and replication, we needed the means of generating high specific activity (P32 labelled) copies of both strands of each gene in a homogeneous form for use as hybridisation probes. The appropriate cloning vectors were available in the Gemini vectors, pGEM1 and PGEM2, and so the first step was to introduce the Rotavirus c-DNA clones into the polylinker region between the SP6 and T7 transcription promotor sites (See Figure 3). In order to minimise errors in the assay and calculation subsequently developed, it was desirable to have the hybridisation probes representing as much of the Rotavirus gene as possible. However, Melton et al (1984) report that for transcription by both SP6 and T7 polymerase the optimum c-DNA insert length is between 200 and 400 base pairs, if the inserted sequence is larger than 400bp then there is a high probability of premature termination of transcription. In fact, as will become clear (See Chapter 2) size of the insert is not the most important factor in cases where incomplete transcription occurs. c-DNA clones of 1kb or less were inserted into the Gemini vectors intact while those larger than this were reduced in size

by excision of an appropriate internal fragment. Following subcloning, the genetic origin and the length of the sub-clones was determined.

RESULTS

A. CLONING OF UKtc ROTAVIRUS RNA

c-DNA clones of all eleven dsRNA segments of the UKtc Bovine Rotavirus had already been generated in pAT153 by McCrae and McCorquodale (1982b and personal communication) using the strategy developed by them.

B. SUBCLONING INTO pGEM1 AND pGEM2

Because of the need to make transcripts as large as possible, c-DNA inserts of approximately 1kb or less were subcloned intact. Clones larger than this were partially subcloned using restriction enzyme sites identified by small scale restriction enzyme digests of the plasmid DNA. The original c-DNA cloning strategy produced clones having a Pst-1 site at either end of the complete insert (McCrae and McCorquodale 1982b), and so for genes 2, and 5 through 11 it was convenient to sub-clone the Pst1-Pst1 fragments directly into the Gemini vectors. The Gene 2 clone had an internal Pst1 site, digestion at which produced a 1kb and a 2kb fragment; the smaller of these was sub-cloned. Gene 10 c-DNA also contained a Pst1 site, in this case both fragments (200 and 400 bp) were subcloned. Fragments of Gene 1 and Gene 3 clones were inserted into double digested pGEM2 at the Pst1-BamH1 and

PstI-HincII sites respectively. Gene 4 c-DNA could be internally cut into two fragments using AccI (Roger Jenkins, personal communication), the larger of these (600bp) was sub-cloned. A summary of the sub-clones generated and subsequently used for transcription is presented in Table 3.

C. VERIFICATION OF CLONE IDENTITY

To check the genetic origin of all the sub-clones generated, 100ng of plasmid DNA was labelled with ^{32}-P dGTP by nick translation and hybridised to "transblot" strips prepared as described in Methods. Comparison of a photograph of the stained gel and the autoradiographs of the hybridised blots allowed verification of the origin of the clones. Separation of Genes 2, 3 and 4 and 7, 8 and 9 required electrophoresis through a 40cm gel for 52 hours, resulting in the loss of Genes 10 and 11. Genes 10 and 11 were therefore analysed separately on a 20cm gel. Figures 4 through 8 show the identification of sub-clones of the eleven Rotavirus daRNA segments.

D. SIZE OF THE SUB-CLONED DNA INSERTS

To determine the size of the sub-cloned inserts 2ug of each sub-clone was digested with the restriction enzyme used for insertion and the fragment released compared to markers of known size. For the smaller (less than 1kb) fragments, markers of pBR322 digested with AluI or HpaII were appropriate. For the

FIGURES 4 to 8: GENETIC ORIGIN OF THE ROTAVIRUS SUB-CLONES

Rotavirus ds-RNA genome was fractionated by PAGE (as described in Materials and Methods) and the gel stained and photographed. RNA was then transferred to DPT paper by electroblotting (Street et al 1982) and probed with radiolabelled subclone DNA. Comparison of Figure 4 with 5 and 6, and of Figure 7 with 8 allows the assignment of the subclones to their gene of origin.

FIGURE 4: POLYACRYLAMIDE GEL OF GENES 1 THROUGH 9

2ug of Rotavirus genomic RNA was loaded onto a 40cm 6% polyacrylamide gel (Laemmli 1970) and electrophoresis at 20mA continued for 52 hours. The gel was stained with Ethidium Bromide and photographed while illuminated by u.v. light. The large arrow indicates the position at which the gel was cut prior to RNA transfer.

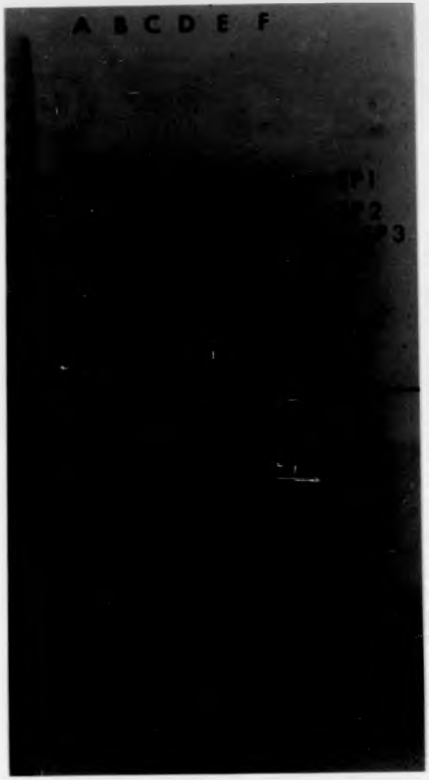

A B C D E F
P1
2
3

FIGURE 5: AUTORADIOGRAPH OF GENOMIC RNA HYBRIDISED TO LABELLED c-DNA CLONES OF GENES 1 THROUGH 4

The upper portion of the RNA gel shown in Figure 4 was transferred to DPT paper and hybridisation was carried out as desribed. Reproduced to original size.

Lane A; Hybridised to clone 6 (Gene 1)

Lane B; Hybridised to clone 8 (Gene 2)

Lane C; Hybridised to clone 33 (Gene 3)

Lane D; Hybridised to clone 11 (Gene 4)

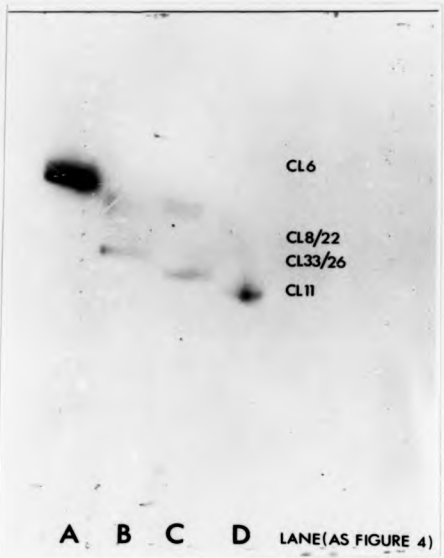

CL6
CL8/22
CL33/26
CL11
A B C D LANE(AS FIGURE 4)

FIGURE 6: AUTORADIOGRAPH OF GENOMIC RNA HYBRIDISED TO LABELLED c-DNA CLONES OF GENES 5 THROUGH 9

The lower portion of the RNA gel shown in Figure 4 was transferred to DPT paper and hybridisation was carried out as decribed. Reproduced to original size.

Lane G; Hybridised to clone 9 (Gene 5)

Lane H; Hybridised to clone 3 (Gene 6)

Lane I; Hybridised to clone 17 (Gene 7)

Lane J; Hybridised to clone 34 (Gene 8)

Lane K; Hybridised to clone 20 (Gene 9)


CL9
CL3
CL17
CL34
CL20
G H I J K LANE (AS FIGURE 4)

FIGURE 7: POLYACRYLAMIDE GEL OF GENES 1 THROUGH 11

2ug of Rotavirus genomic RNA was loaded on a 20cm 6% polyacrylamide gel (Laemmli 1970) and electrophoresis at 20mA continued for 16 hours. The gel was stained with Ethidium Bromide and photographed while illuminated by u.v. light.

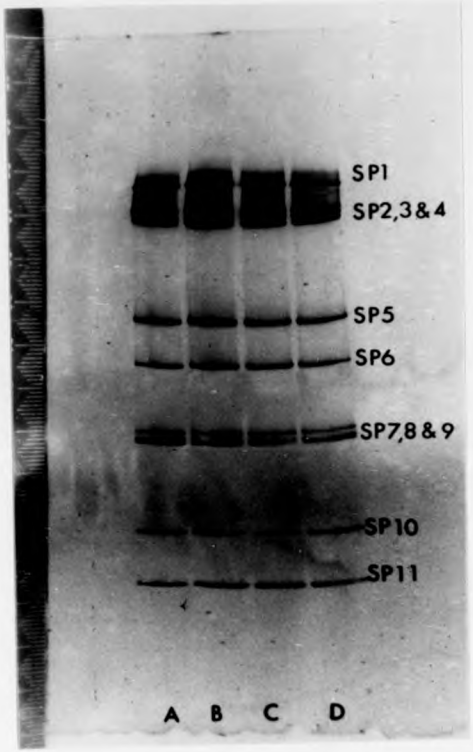
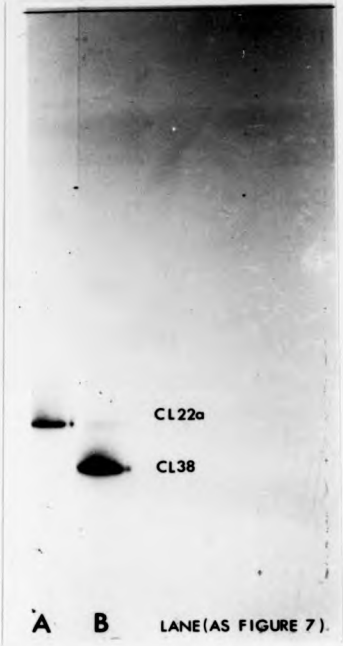

SP1
SP2,3&4
SP5
SP6
SP7,8&9
SP10
SP11
A B C D

FIGURE 8: AUTORADIOGRAPH OF GENOMIC RNA HYBRIDISED TO LABELLED c-DNA CLONES OF GENES 10 and 11

The RNA gel shown in Figure 4 was transferred to DPT paper and hybridisation was carried out as decribed. Reproduced to original size.

Lane A; Hybridised to clone 22a(Gene 10)

Lane B; Hybridised to clone 38 (Gene 11)


CL22a
CL38
A
B
LANE(AS FIGURE 7).

larger inserts (900-1150 bp) Phage Lambda DNA digested with both ECoR1 and HinDIII, and pBR322 digested with AvaII and BstN1 were used. Figures 9 and 10 show the released inserts compared to the markers in 1.5% agarose gels (cast and run in 1 X Loenings buffer). The approximate sizes of the 11 sub-clones are shown in Table 3.

SUMMARY

Since c-DNA clones to eight of the eleven Rotavirus genes had already been generated at the inception of this work, it was straight-forward to sub-clone the whole or a part of each into the pGEM1 and pGEM2 transcription vectors. Most of the clones were inserted at the Pst1 site of the polylinker region. For the Gene 2 sub-clone it was necessary to generate clones containing the c-DNA inserted in opposite orientations. Reference to Figure 3 (and legend) shows that since the Gene 2 c-DNA was inserted at the Pst1 site, there was only one restriction enzyme site available for linearisation of the plasmid for transcription with SP6 polymerase (in pGEM2); HindIII. The Gene 2 c-DNA contains an internal HinDIII site, and so digestion and subsequent transcription by SP6 would produce only a partial copy of the c-DNA from the 5' end to the HinDIII site. By inserting the c-DNA in the reverse orientation it was possible to copy both strands of the clone using T7 polymerase using SalI for linearisation in both cases.

FIGURES 9 AND 10: SIZE ANALYSIS OF THE c-DNA INSERTS SUB-CLONED INTO pGEM1 AND pGEM2

2ug of each sub-clone was digested with the restriction enzyme(s) used for insertion, and electrophoresis carried out in 1.5% agarose gels together with marker DNA fragments.

FIGURE 9: Electrophoresis of digested pGEM vectors containing Gene 1,2,6,7,9,10 and 11 c-DNA inserts and pBR322 digested with Hpa11 or Alu1.

Lane A; Alu1 digest of pBR322

Lane B; pGEM2 containing Gene 1 c-DNA

Lane C; pGEM1 containing Gene 4 c-DNA

Lane D; pGEM1 containing Gene 6 c-DNA

Lane E; pGEM1 containing Gene 7 c-DNA

Lane F; pGEM2 containing Gene 9 c-DNA

Lane G; pGEM1 containing Gene 10 c-DNA

Lane H; pGEM1 containing Gene 11 c-DNA

Lane I; Hpa11 digest of pBR322


A B C D E F G H I

FIGURE 10: Co-electrophoresis of digested pGEM vectors containing Gene 2,3,5 and 8 c-DNA inserts, Lambda DNA digested with HinD111 and ECoR1, and pBR322 digested with Aval1 of BstN1.

Lane A; pGEM2 containing Gene 2 cDNA

Lane B; pGEM2 containing Gene 3 cDNA

Lane C; pGEM1 containing Gene 5 cDNA

Lane D; pGEM1 containing Gene 8 cDNA

Lane E; Lambda DNA digested with HinD111 and ECoR1

Lane F; Aval1 digest of pBR322

Lane G; BstN1 digest of pBR322


1375
1060
947
928
831
A B C D E F G

TABLE 3: CHARACTERISTICS OF ROTAVIRUS SUBCLONES IN pGEM 1 AND pGEM 2.

Table 3 summarises the data presented in Chapter 1. c-DNA clones to all 11 Rotavirus genes were inserted into the polylinker region of pGEM1 or pGEM 2. The restriction enzyme site(s) used and the size of the inserted c-DNA (from Figures 9 and 10) is given for each clone.

GENE	VECTOR	INSERTION SITE	SIZE OF INSERT (base pairs)	CLONE NUMBER
1	GEM2	PST1-BAMH1	570	CLONE 6
2	GEM2	PST1	800	CLONE 8
2	GEM2	PST1	800	CLONE 22
3	GEM2	PST1-HINCII	1150	J25/CLONE 33
3	GEM1	HINCII-PST1	1150	J25/CLONE 26
4	GEM1	ACCI	600	CLONE 11
5	GEM1	PST1	800	CLONE 9
6	GEM1	PST1	500	CLONE 3
7	GEM1	PST1	600	CLONE 17
8	GEM1	PST1	1150	CLONE 34
9	GEM2	PST1	670	CLONE 20
10	GEM1	PST1	240	CLONE 22a
11	GEM1	PST1	650	CLONE 38

At the commencement of this work, c-DNA clones to genes 1,3 and 4 had not been isolated. However a c-DNA library of the Rotavirus genome had been prepared (McCrae personal communication) and the author was able to identify c-DNA copies of these genes from this. The c-DNAs identified were 2.8 (Genes 1 and 3) and 3.0 (Gene 4) Kb in length, and so smaller fragments were excised prior to ligation into the pGEM1 and pGEM2 vectors as previously described.

RESULTS: CHAPTER 2.

CHAPTER 2

IN VITRO TRANSCRIPTION USING pGEM1 AND pGEM2 CONTAINING ROTAVIRUS cDNA SEQUENCES.

INTRODUCTION

Having sub-cloned the Rotavirus cDNA sequences into the Gemini Vectors it was then possible to generate RNA copies of both strands of the genes. A modification of the method recommended by Promega Biotec was finally used for the Transcription reactions which were of two types:

1) "Cold" or low specific activity RNA transcripts;

Stocks of "unlabelled" transcripts were prepared using saturating amounts of all four ribonucleotides (10uCi ^{3}-H UTP was included to facilitate calculation of RNA concentration after synthesis). These transcripts which represented each strand of the 11 genes were for use as "standards" in the hybridization assay described in Chapter 3. The "cold" reactions which yielded upto 15ug of transcript per reaction had only to be performed once, to give a stock of RNA.

2) "Hot" or high specific activity transcripts.

High specific-activity transcripts were generated by replacing the cold rCTP with ^{32}-P rCTP (800Ci/mMol). These transcripts were used as probe in the hybridisation assay and fresh RNA was transcribed each time a hybridisation was carried out.

Each transcript was analysed to verify that the whole of the cDNA insert was being copied during transcription, since premature termination of transcription in either direction would result in poor or absent hybridisation of the two complementary RNA strands. It was also necessary to determine the orientation of the inserts with respect to the transcriptase promotors; by using dot-blot hybridisation it was possible to determine the "sense" (mRNA sense (+) or complementary sense (minus-)) of each of the transcripts. Furthermore, in order to make the resulting hybridisation assay quantitative it was necessary to determine the actual length of the ds-RNA hybrids formed when the "probe" transcripts were hybridized to the infected cell RNA (see Chapter 3), this was achieved by hybridising transcripts to Rotavirus genomic and messenger RNA and treating the hybrids with exactly the same conditions as used in the final assay, with size analysis by PAGE.

RESULTS

A. IN VITRO TRANSCRIPTION

In order to obtain run-off transcripts of defined length, plasmid DNA was first linearised at a restriction enzyme site downstream of the inserted c-DNA (distal to the polymerase promotor being used, see Figure 3), and which was not contained within the insert sequence. Only the Gene 2 clone presented a problem in this respect as described in Chapter 1. Most clones were linearised using SalI and HinDIII, although in some cases it was necessary to digest with a restriction enzyme such as PstI or

SacI which generates a 3' protruding end or "overhang". In cases where this could not be avoided for various reasons, spurious transcripts thought to be due to adventitious transcription by the polymerase on the opposite DNA strand were observed (Promega 1985). To eliminate this the PstI and SacI ends were treated with Mung Bean Nuclease (Maniatis et al 1982) which removes the protrusion.

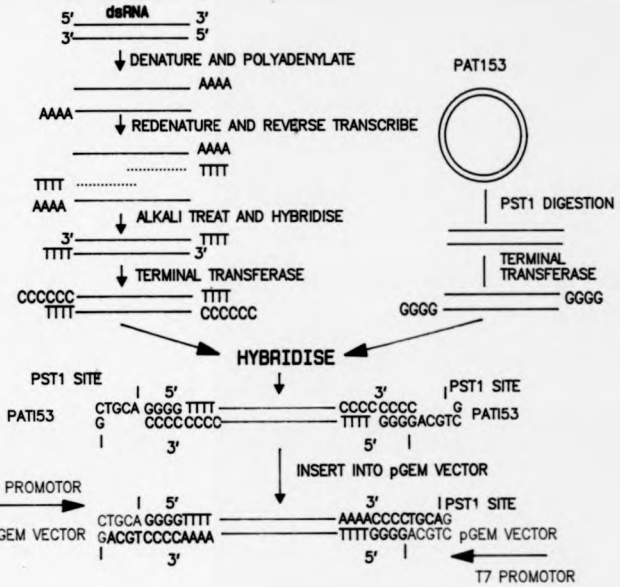
The in-vitro transcription reaction was carried out as described in Materials and Methods. It was important to ensure freshness of all the ingredients especially the rGTP stock and to set up the reaction at room temperature to avoid the precipitation of the DNA template by the spermidine in the transcription buffer (this often takes place at low temperature). Typically 25-50uCi of -PrCTP was used in the generation of high specific activity transcript, and 60% or more of the label was incorporated into TCA precipitable material. Although 40°c is the recommended temperature for SP6 and T7 polymerase activity, 37°c was used for the reactions, for the following reason; At 40°c the total incorporation of -PrCTP was at a higher level but the proportion of incomplete transcripts (estimated by agarose gel electrophoresis) was higher than that at 37°c. Transcription at 30°c was also examined, but at this lower temperature the level of incorporation was much reduced, and 37°c was therefore used for transcription as a compromise between reduced incorporation and the production of many truncated transcripts. The effect of increasing the incubation time from 1 to 2 hours was also examined. This did increase incorporation of label by 15-20% but

this seemed to be mainly due to synthesis of truncated transcripts and therefore a synthesis time of 1hr was routinely used.

It was found that only ^{32}-PrCTP or rATP (and not rGTP or rUTP) could be used for transcription of high specific activity RNA. In the original cloning strategy, (McCrae and McCorquodale 1982b) the plasmid (pAT153) DNA was tailed with multiple G residues, to allow ligation to insert c-DNA tailed with multiple C residues (by terminal transferase, see also Figure 11). As a result the inserted c-DNA is flanked by a region of homopolymer tails; G and T at the 5' end of the c-DNA, and A and C at the 3' end (see Figure 11). The Pst1 sites contructed during this procedure are on the plasmid side of these tails and so sub-cloning using Pst-1 digestion results in both the inserted c-DNA and the homopolymer tails being transferred into the Gemini vectors (Figure 11). Transcription from either the SP6 or the T7 polymerase promotor entails polymerisation through Poly G and Poly T before the c-DNA sequence is reached. The use of rGTP or rUTP as the limiting ribonucleotide thus leads to extremly poor strand elongation, and so ^{32}p-rCTP was used for transcription of the "hot" RNA. There is a region of Poly C at the 3' end of most of the clones and termination of elongation within this region due to substrate limitation may lead to slight size heterogenity in the transcripts. However, this is not important since this region will not hybridise to the Rotavirus RNA in subsequent hybridisation assays and will be removed by the RNases (see Chapter 3).

FIGURE 11: THE MOLECULAR CLONING OF dsRNA OF ROTAVIRUS AND INSERTION OF c-DNA INTO THE GEMINI VECTORS.

The cloning strategy for dsRNA developed by McCrae and McCorquodale (1982) is reproduced to illustrate the structure of the c-DNA. As a result of the G and C residues added to pAT153 and c-DNA respectively, the c-DNA is flanked by a region of homopolymer tails. External to these are the recreated Pst-1 sites, when c-DNA is excised from pAT153 and ligated into the Gemini vectors the homopolymer tracks are placed downstream of the polymerase promotor sites.


5′ dsRNA 3′
3′ 5′
DENATURE AND POLYADENYLATE
AAAA
AAAA
REDENATURE AND REVERSE TRANSCRIBE
AAAA
TTTT
TTTT
AAAA
ALKALI TREAT AND HYBRIDISE
3′ TTTT
TTTT 3′
TERMINAL TRANSFERASE
CCCCCC TTTT
TTTT CCCCCC
PAT153
PST1 DIGESTION
TERMINAL TRANSFERASE
GGGG GGGG
HYBRIDISE
PST1 SITE
PAT153
CTGCA GGGG TTTT CCCC CCCC G
G CCCC CCCC TTTT GGGG ACGTC
PAT153
PST1 SITE
3′ 5′
5′ 3′
INSERT INTO pGEM VECTOR
SP6 PROMOTOR
pGEM VECTOR
CTGCA GGGGTTTT AAAACCCCTGCAG
GACGTCCCCAAAA TTTTGGGGACGTC
pGEM VECTOR
PST1 SITE
3′ 5′
5′ 3′
T7 PROMOTOR

Initial examination of the transcripts was carried out in denaturing agarose gels (MOPS/formaldehyde) and any clones generating transcripts of heterologous size were rejected. This was necessary in two cases; The 400bp subclone of Gene 10 gave rise to T7 transcripts of three defined lengths, one appeared to represent a full length copy of the cDNA, and the other two were approximately 100 and 200 bp in length. Transcription of the 200bp fragment generated homogeneous transcripts (see Figure 15) and was therefore used subsequently. Genes 3 and 5 also presented difficulties: The Gene 3 clone (J25/CL33) was excised from pGEM2 and re-ligated into pGEM1, since it was fully transcribed in only one direction; by moving it from one vector to the other it was effectively turned round, and full length transcripts of both strands could be obtained using T7 polymerase. A Gene 5 clone of 1.2Kb yielded transcripts of only approximately 500bp in length on transcription with T7 polymerase, while a smaller clone (700bp Clone 9, Table 3) yielded complete transcripts. Figures 12 to 15 show examples of transcripts generated. All transcripts were composed predominantly of full-length copies of the cloned DNA and the SP6 and T7 transcripts for each gene were of the same length confirming that the inserted c-DNA was being fully copied in both directions. Figure 12 shows an example of transcription of a template having a 3' protrusion (SacI digested). In track A a truncated transcript is visible (approximately 300bp), the result of transcribing the Gene 5 subclone linearised with SacI. When a SalI linearised template was used in the same reaction this extra transcript was absent.

FIGURES 12 TO 15: AGAROSE GEL ELECTROPHORESIS OF HIGH SPECIFIC ACTIVITY TRANSCRIPT RNA.

Transcripts were analysed initially on 1.5% agarose denaturing MOPS/formaldehyde gels. Electrophoresis was carried out in 1 X MOPS buffer at 50mA for 8-16 hours depending on the size of the transcript being analysed. A radiolabelled ECoR1/HinDIII digest of Lambda DNA was used as size marker and fragment lengths in base pairs are indicated on the figures. "w" represents the origin of migration. The small differences in length between the SP6 and T7 transcripts of several of the genes is due to the length of polylinker sequence transcribed preceeding the gene. This varies depending on the polymerase being used since the Pst-1 site most often used for insertion of the c-DNA is not central.

FIGURE 12: ANALYSIS OF RNA TRANSCRIPTS FOR GENES 5 AND 6.

Lanes A,D and G; ECoR1/HinDIII digest of Lambda DNA labelled by Klenow filling in the presence of 32-P dGTP.

Lane B; Gene 5 DNA template transcribed with SP6 polymerase.

Lane C; Gene 5 DNA template transcribed with T7 polymerase.

Lane E; Gene 6 DNA template transcribed with SP6 polymerase.

Lane F; Gene 6 DNA template transcribed with T7 polymerase.

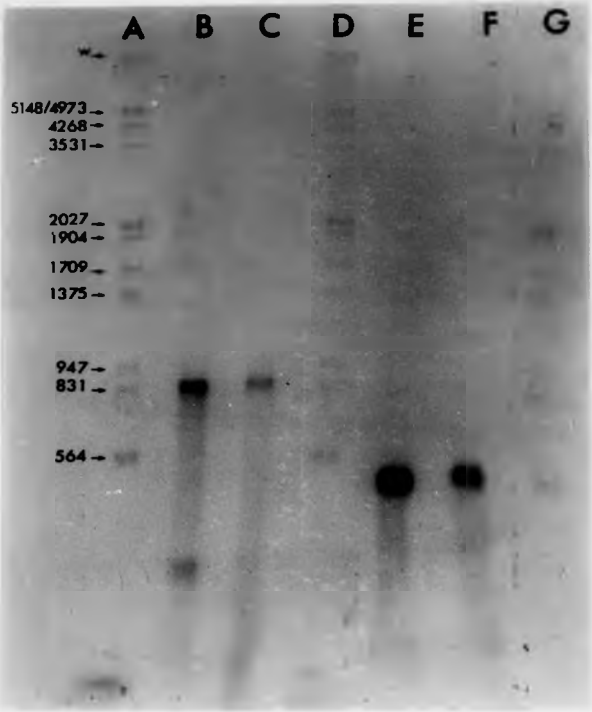

A
B
C
D
E
F
G
w
5148/4973
4268
3531
2027
1904
1709
1375
947
831
564

FIGURE 13: ANALYSIS OF RNA TRANSCRIPTS FOR GENES 7 AND 8.

Lanes A,D and G; ECoR1/HinDIII digest of Lambda DNA labelled by Klenow filling in the presence of 32-P dGTP.

Lane B; Gene 7 DNA template transcribed with SP6 polymerase.

Lane C; Gene 7 DNA template transcribed with T7 polymerase.

Lane E; Gene 8 DNA template transcribed with SP6 polymerase.

Lane F; Gene 8 DNA template transcribed with T7 polymerase.

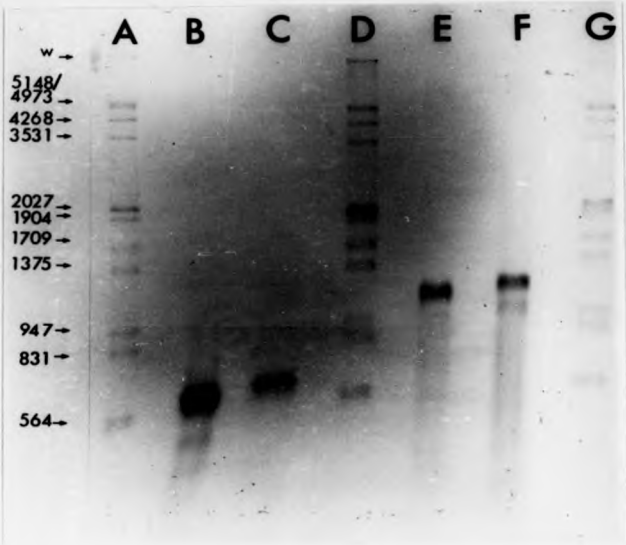

A
B
C
D
E
F
G
w
5148/
4973
4268
3531
2027
1904
1709
1375
947
831
564

FIGURE 14: ANALYSIS OF RNA TRANSCRIPTS FOR GENE 9.

Lanes A, and D; ECoR1/HinDIII digest of Lambda DNA labelled by Klenow filling in the presence of 32-P dGTP.

Lane B; Gene 9 DNA template transcribed with SP6 polymerase.

Lane C; Gene 9 DNA template transcribed with T7 polymerase.

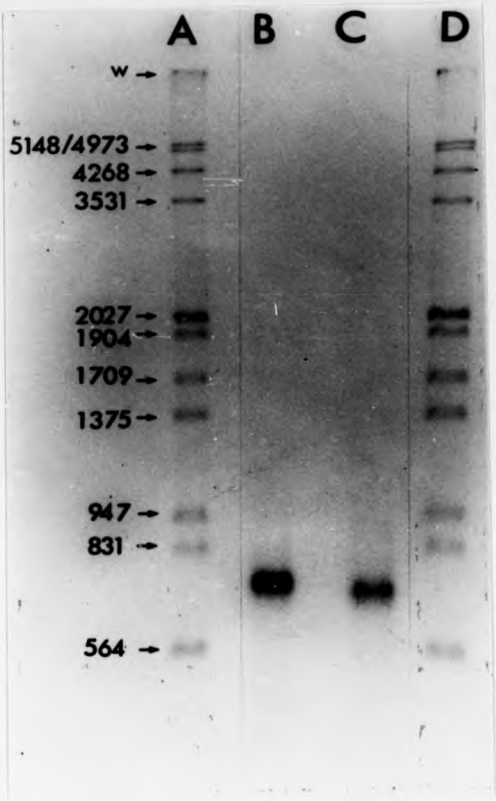

A
B
C
D
w
5148/4973
4268
3531
2027
1904
1709
1375
947
831
564

FIGURE 15: ANALYSIS OF RNA TRANSCRIPTS FOR GENE 10.

Lanes A, and D; ECoR1/HinDIII digest of Lambda DNA labelled by Klenow filling in the presence of 32-P dGTP.

Lane B; Gene 10 DNA template transcribed with SP6 polymerase.

Lane C; Gene 10 DNA template transcribed with T7 polymerase.


A
B
C
D
w
5148/4973
4268
3531
2027/1904
1709
1375
947
831
564
125

B. CHARACTERIZATION OF THE RNA TRANSCRIPTS

1. ORIENTATION

In order to determine which strand of the RNA duplex was represented by the transcripts, samples were hybridised to Nylon membrane loaded with decreasing amounts of Rotavirus mRNA and ds-genomic RNA (applied by Dot-Blotting). Figure 16 shows the results obtained for the Gene 1 subclone. The T7 transcript is able to hybridise to both the mRNA and the dsRNA indicating that it is complementary to message and is therefore minus-sense. The orientation of the insert is confirmed by an identical blot hybridised to the SP6 transcript, which is of the same sense as mRNA, plus-sense and therefore gives a signal only with genomic dsRNA. Such Blots were prepared for all the genes. Figures 17 and 18 show a summary of this data, in this case the "cold" RNA transcripts have been applied to the nylon and labelled mRNA or dsRNA used as the probe. In Figure 17 only those transcripts being minus-sense hybridise to labelled mRNA while all transcripts hybridise to labelled dsRNA (Figure 18). It is not known why these signals vary in intensity.

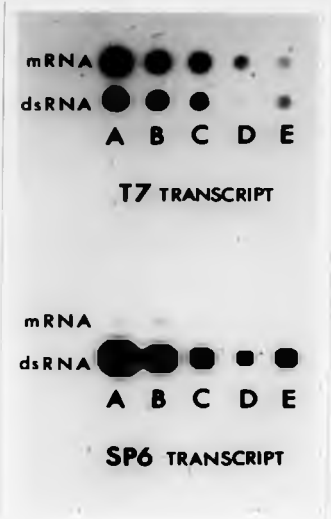
2. SIZE OF dsRNA HYBRIDS FORMED BY THE TRANSCRIPTS

To accurately assess the length of the hybrids formed by the transcripts during the hybridisation assay, reactions were set up in which 500,000 cpm of high Specific Activity RNA transcript was hybridised to 100ng of complementary RNA. The hybridisation and subsequent RNase treatment was

FIGURE 16: DETERMINATION OF TRANSCRIPT ORIENTATION BY DOT-BLOT ANALYSIS.

Initial determination of the "sense" of each transcript was carried out by hybridisation to replicate Dot-Blots loaded with Rotavirus dsRNA and mRNA.
Nylon membranes were loaded as follows:
Messenger RNA: A) 1ug B) 500ng C) 100ng D) 50ng E)10ng
DS (Genomic) RNA: A) 1ug B) 500ng C) 100ng D) 50ng E)10ng

Figure 16 shows the results obtained with Gene 1(clone 38): Hybridisation of the T7 transcript to both dsRNA and mRNA indicates that it is "minus-sense", while hybridisation of the SP6 transcript to dsRNA only indicates that it is "plus-sense".


mRNA
dsRNA
A B C D E
T7 TRANSCRIPT
mRNA
dsRNA
A B C D E
SP6 TRANSCRIPT

FIGURES 17 AND 18: SUMMARY OF THE "SENSE" OF THE RNA TRANSCRIPTS.

10ng of each cold transcript was applied to nylon membrane by Dot-Blotting as follows:

ROW A:	1)	Gene 1,	Clone 38	SP6 transcript.
	2)	Gene 2,	Clone 8	T7 transcript.
	3)	Gene 3,	Clone J25/33	T7 transcript.
	4)	Gene 4,	Clone 11	SP6 transcript.
	5)	Gene 5,	Clone 9	SP6 transcript.
	6)	Gene 6,	Clone 3	SP6 transcript.
	7)	Gene 7,	Clone 17	SP6 transcript.
	8)	Gene 8,	Clone 34	SP6 transcript.
	9)	Gene 9,	Clone 20	SP6 transcript.
	10)	Gene 10,	Clone 22a	SP6 transcript.
	11)	Gene 11,	Clone 6	SP6 transcript.

ROW B:	1)	Gene 1,	Clone 38	T7 transcript.
	2)	Gene 2,	Clone 22	T7 transcript.
	3)	Gene 3,	Clone J25/26	T7 transcript.
	4)	Gene 4,	Clone 11	T7 transcript.
	5)	Gene 5,	Clone 9	T7 transcript.
	6)	Gene 6,	Clone 3	T7 transcript.
	7)	Gene 7,	Clone 17	T7 transcript.
	8)	Gene 8,	Clone 34	T7 transcript.
	9)	Gene 9,	Clone 20	T7 transcript.
	10)	Gene 10,	Clone 22a	T7 transcript.
	11)	Gene 11,	Clone 6	T7 transcript.

FIGURE 17: Membrane probed with Kinase labelled Rotavirus messenger RNA. Only transcripts of a minus-sense orientation hybridise to the probe RNA.

FIGURE 18: Membrane probed with Kinase labelled Rotavirus genomic RNA (ds-RNA). All transcripts hybridise to the probe RNA.


A
1 2 3


A

PAGINATION
ERROR

carried out exactly as in the final hybridisation assay conditions used. Following phenol extraction and ethanol precipitation hybrids were analysed on non-denaturing 5% polyacrylamide gels alongside size markers. Figures 19 to 21 illustrate the type of results obtained. Table 4 summarises the data obtained for all genes by this type of analysis.

DISCUSSION

After modification of the transcriptase reaction it was possible to efficiently generate homogeneous "cold" and high Specific Activity copies of the cloned cDNA inserts. Cold transcripts were examined on denaturing 8M Urea 5% polyacrylamide gels to check for homogeneity, however due to the poor staining of single stranded RNA in these gels it was not possible to make good photographic records. The homogeneity achieved with high specific activity transcripts has been illustrated in Figures 12 to 15.

It was noted, despite the recommendation of Melton et al (1984) that cDNA upto 1kb in length could be transcribed efficiently. It would seem that complete transcription is dependent upon the structure of the insert DNA itself since the Gene 8 and 3 sub-clones could be copied in their entirety (>1kb) whereas a very much smaller subclone for Gene 10 (400bp) appeared to contain two sites at which T7 polymerase transcription was terminated. These "sites" could take the form of secondary structures such as hairpin loops, preventing polymerase passage along the DNA and causing premature termination of strand elongation. The truncated transcripts produced from such larger

FIGURES 19 TO 21: POLYACRYLAMIDE GEL ANALYSIS OF dsRNA HYBRIDS FORMED BETWEEN TRANSCRIPT AND ROTAVIRUS GENOMIC AND MESSENGER RNA.

Figures 19 to 21 illustrate the analysis of dsRNA hybrids composed of a high specific activity transcript and Rotavirus RNA, treated as described in Materials and Methods, and fractionated on 5% non-denaturing polyacrylamide gels. Following electrophoresis gels were dried and exposed for autoradiography.

FIGURE 19: ANALYSIS OF THE HYBRIDS FORMED BETWEEN TRANSCRIPTS FOR GENE 3 AND ROTAVIRUS RNA.

Electrophoresis was carried out on a 40cm Gel at 30mA for 48 hours.

Size markers: An ECoR1/HinDIII digest of Lambda DNA was labelled with 32-P dGTP using the Klenow fragment of DNA polymerase I, to form markers of known size; these are shown on the far left of Figure 19 with their size in base pairs indicated.

Lane A: T7 transcript of Gene 3 Sub-clone J25/26 hybridised to Rotavirus mRNA.

Lane B: T7 transcript of Gene 3 Sub-clone J25/33 hybridised to Rotavirus dsRNA.

Accurate sizing of this hybrid was achieved by measurement of the positions of Hinf1, AvaII and BstN1 digests of plasmid pBR322 DNA on the gel stained with ethidium bromide. Unfortunately the bands were too feint to allow a clear photographic record to be made.

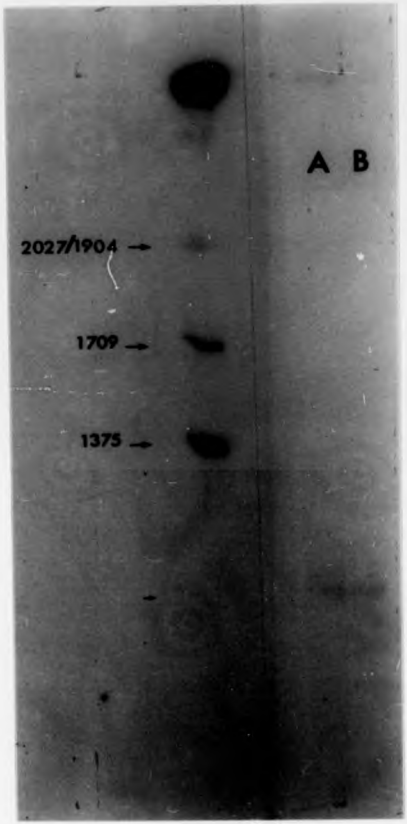

A B
2027/1904
1709
1375

FIGURE 20: ANALYSIS OF THE HYBRIDS FORMED BETWEEN TRANSCRIPTS FOR GENE 6 AND ROTAVIRUS RNA AND INFECTED CELL RNA.

Electrophoresis was carried out on a 20cm gel for 6 hours at 30mA.

Size markers: A HpaII digest of pBR322 DNA was labelled using Klenow as previously described, these are shown on the left of the figure with size in base pairs indicated.

Lane A: An SP6 transcript of the Gene 6 sub-clone hybridised to infected cell RNA collected at 6.5 hours post-infection.

Lane B: An SP6 transcript of the Gene 6 Sub-clone hybridised to Rotavirus mRNA.

Lane C: A T7 transcript of the Gene 6 Sub-clone hybridised to Rotavirus Genomic RNA.

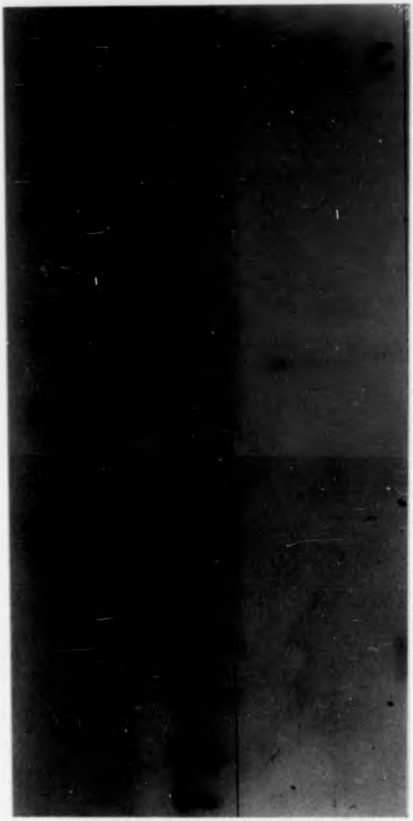


FIGURE 21: ANALYSIS OF THE HYBRIDS FORMED BETWEEN TRANSCRIPTS FOR GENE 11 AND ROTAVIRUS RNA.

Electrophoresis was carried out on a 20cm gel for 6 hours at 30mA.

Size markers: A HpaII digest of pBR322 DNA was labelled using Klenow as previously described, these are shown on the left of the figure with size in base pairs indicated.

Lane A: An SP6 transcript of the Gene 11 sub-clone hybridised to Rotavirus mRNA.

Lane B: A T7 transcript of the Gene 11 sub-clone hybridised to Rotavirus Genomic RNA.

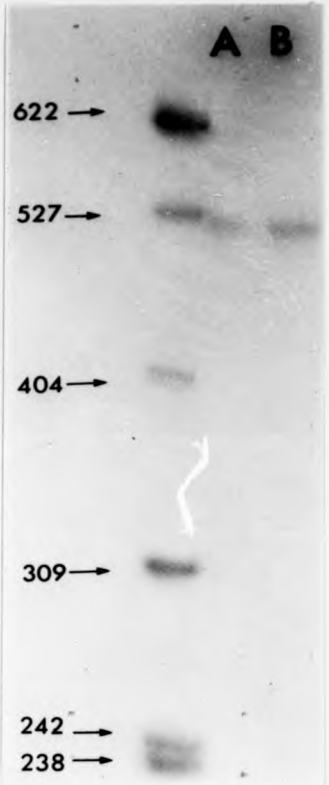

A
B
622
527
404
309
242
238

TABLE 4: CHARACTERISTICS OF THE RNA TRANSCRIPTS USED IN HYBRIDISATION ASSAYS.

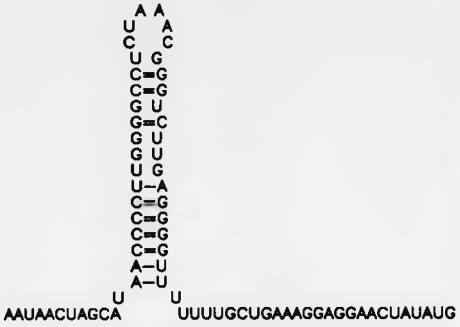
Table 4 summarises the data presented in Chapter 2. Note that SP6 and T7 polymerase were used to transcribe one subclone to generate copies of both strands of the c-DNA in 9 of the 11 genes. For genes 2 and 3, separate subclones containing the c-DNA on opposite orientations were transcribed with T7 polymerase. The sizes of the hybrids formed with complementary RNA under the conditions described are shown in the final column and are the figures used in subsequent calculations.

GENE	CLONE	FOR T7 LINEARISE WITH	"SENSE"	FOR SP6 LINEARISE WITH	"SENSE"	SIZE OF HYBRID
1	38	BAMHI	MINUS	PSTI	PLUS	430bp
2	8	SALI	MINUS			640bp
2	22	SALI	PLUS			640bp
3	J25/33	HINCII	PLUS			1070bp
3	J25/26	PSTI	MINUS			1070bp
4	11	HINDIII	MINUS	BAMHI	PLUS	530bp
5	9	HINDIII	PLUS	SALI	MINUS	640bp
6	3	HINDIII	PLUS	SALI	MINUS	370bp
7	17	HINDIII	PLUS	SALI	MINUS	400bp
8	34	HINDIII	MINUS	SALI	PLUS	990bp
9	20	SALI	MINUS	HINDIII	PLUS	520bp
10	22A	HINDIII	PLUS	SALI	MINUS	200bp
11	6	HINDIII	PLUS	SALI	MINUS	520bp

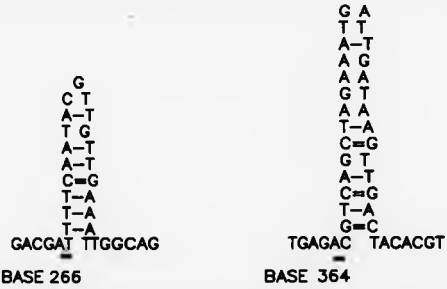
clones are thought to be due to secondary structures within the DNA sequence incompatible with transcription (Melton et al 1984). To investigate this possibility, the sequence of the 400bp Gene 10 clone was searched for matches with the T7 termination sequence (a hairpin-loop structure) as published by Dunn and Studier (1983 and Figure 22) using Stadens "Analyseq" software. At two positions on the coding (plus-sense) strand and 3 positions on the complementary strand the Gene 10 subclone showed a 45% homology with with the T7 terminator sequence, and at one position on the complementary strand there was a 50% homology. Since the T7 terminator consists of a hairpin loop formed by base pairing, the Gene 10 homologous sequences were examined for residues which could form such a stem-loop structure. Of the 6 sequences identified 3 had only 1 such pairing, 2 had two, and 1 had three base-pairs in the potential stem. It is therefore doubtful given the low level of homology with the T7 terminator sequence that any of these sequences could function as T7 terminators. The Gene 10 sequence was then examined for potential hairpin-loop structures, of which there were several. Figure 22 illustrates the two stem-loops identified on the plus-sense strand (the T7 transcript of the clone was minus-sense) of the c-DNA which had a high number (7) of base-pairs in the stem of the structure and which occurred at positions 266 and 364, corresponding to 159 and 257 base-pairs from the transcription start site (this sub-clone started at position 107 of the Gene and ended at position 557). Termination of transcription at these two sites could have produced the truncated transcripts observed, although a fuller understanding of the requirements of the T7 polymerase is needed before a firm conclusion can be reached.

FIGURE 22: THE STRUCTURE OF THE T7 TERMINATION SIGNAL, AND POTENTIAL HAIRPIN-LOOP STRUCTURES IN THE GENE 10 (400bp) SUBCLONE.

When the 400bp subclone of Gene 10 was transcribed with T7 polymerase, transcripts of three defined lengths were observed. Since the sequence of the T7 termination signal is known it was possible to search the Gene 10 sequence for matching sequences, however none were found which could account for the premature termination of transcription. The Gene 10 subclone sequence which extends from base 107 to 557 was then examined for potential hairpin-loop structures bearing a resemblence to the T7 termination sequence. Figure 22 shows two of these which, if capable of causing premature termination, would yield transcripts of approximately the correct length (see text).



T7 TERMINATOR SEQUENCE



POTENTIAL HAIRPIN–LOOP STRUCTURES
IN GENE 10 (POSITIONS 107–557)

It will be noted from comparison of Tables 3 and 4, that the length of the dsRNA hybrids was, on average 130bp shorter than that estimated for the length of the DNA insert. This difference may be accounted for by the presence of the A-T and G-C tails introduced during cloning (see Part A, this Chapter). Since these bases are internal to the PstI site all sub-clones except that for Gene 4 contains at least one set of these tails and comparison of Figures 9 and 10 with Figures 12 to 15 shows that the RNA transcripts were the same length as the corresponding c-DNA. When such transcripts were hybridised to complementary RNA sequences, these tails remained unpaired and were therefore removed by the RNase digestion. The differential reduction in size between the various hybrids may reflect the length of the A-T and G-C tails and also the strength of base-pairing at the termini of the hybrids; for example if base pairing is weak at the termini (perhaps due to a high proportion of A-T base pairs) then hybrids may be "nibbled" back by the RNases. These sizes reflect the true extent of hybridisation during the assay and they are therefore used in the calculation shown in Chapter 4.

RESULTS: CHAPTER 3.

CHAPTER 3

DEVELOPMENT OF A SOLUTION HYBRIDISATION ASSAY

INTRODUCTION

Having prepared reagents with which to develop a hybridisation assay there were several approaches available to us. The strand specific probes could have been applied to a Dot-Blot assay, such as that used by Piwnica-Worms and Keene (1985) for Vesicular Stomatitis Virus. This approach would involve applying experimental samples to Nylon or Nitrocellulose filters, probing with labelled RNA transcript followed by autoradiography and excision of the dots for counting. Although Piwnica-Worms and Keene found such as approach satisfactory, and were able to construct an appropriate calibration system, we decided to develop a solution-based assay, since it was felt that this would allow more direct quantitation and the detection of smaller amounts of viral RNA. As a starting point for the assay the method for Solution Hybridisation and RNase (S1 Type) mapping described in the Promega Biotec Technical Bulletin 002 was used. Following treatment with RNase, this method recommends the analysis of hybrid molecules by PAGE in 8M Urea gels. Although this approach was open to us, the autoradiographs would then have to be analysed by densiometry in order to make the assay quantitative. In addition, once it had been established that the hybrids in question were of a defined length and homogeneous in size, direct

visualisation by such a procedure was unnecessary. We therefore decided to collect the RNase resistant material by TCA precipitation. All of the modifications described in this Chapter were intended to reduce the variability between repeat samples, and to increase the sensitivity and accuracy of the assay.

RESULTS

Initial refinement of the solution hybridisation and subsequent RNase treatment of the hybridized material was carried out using a "medium" specific activity (170Ci/mMol) minus-sense transcript of Gene 8 and a range of Rotavirus mRNA concentrations. Briefly the starting procedure was as follows; Rotavirus messenger RNA samples were suspended in 30ul of 80% Formamide hybridisation buffer. High specific activity transcript (probe) was resuspended in 100ul of the same buffer following ethanol precipitation. 1ul of the probe RNA was then added to each of the mRNA samples in 0.5ml Eppendorf tubes which were heated to 85°c for 10 minutes. Hybridisation was allowed to proceed at 42°c for 8-16 hours. The 30ul hybridisation reaction was then transferred to a 1.5ml Eppendorf tube and 300ul of RNase buffer containing 40ug/ml RNase A and 2ug/ml RNase T1 was added and digestion allowed to proceed for 1 hour at 30°c. The reaction was terminated by the addition of 20ul of 10% SDS (final concentration 0.85%) and 50ug Proteinase K with incubation for 15 minutes at 37°c. The total sample was then applied to strips of Whatmann 3MM paper which were washed twice in 5% TCA for 10 minutes and 5 minutes, rinsed twice with 100% ethanol, twice with diethly-ether and air dried prior to counting in Beckman EP liquid scintillant.

The first data obtained using this procedure showed a variability of upto 100% between duplicate samples. Several alterations to the procedure were made with the aim of decreasing this variability.

A. RESUSPENSION OF RNA

Following desiccation, "probe" RNA was resuspended in 100ul of formamide hybridisation buffer by the sequential addition of 10ul water, 10ul 10 X PIPES buffer and 80ul 100% deionised formamide pH6.7. This sequential addition ensured complete resuspension of the probe RNA which was then diluted appropriately and added to the hybridisation in a volume of 10ul. The total volume of the hybridisation was maintained at 30ul.

B. TIME AND TEMPERATURE OF HYBRIDISATION

I. TIME

It was found that beyond 16 hours incubation there was no further improvement in the extent of hybridisation, Infact by 24 hours the amount of RNase resistant material in hybridisations had fallen to approximately 75% of the amount present at 16 hours (Refer to Table 7 row 2 or 3, by which time RNase digestion is complete). This was probably due to radiolytic degradation of probe and hybrid during the prolonged incubation. The level of RNase resistance seen after 8 hours incubation was similar to that at 16 hours, which was used routinely.

II. TEMPERATURE

42° c is the optimum temperature for filter hybridisation with Rotavirus RNA and cDNA (Padley, personal communication). Solution hybridisations were performed at 15, 30, 37, 42, and 50°c, with a Gene 8 minus sense transcript and 20ng of messenger RNA (a molar excess to ensure saturation of the probe). Although the buffer used in filter hybridisations is different to the Formamide hybridisation buffer, the results shown in Table 5 indicate that between 37 and 42° c is the preferred temperature.

C. RNASE DIGESTION

Digestion of the single stranded RNA remaining after hybridisation was carried out with RNase A and RNase T1 using conditions under which they are specific for single-stranded RNA.

I. SETTING UP THE DIGESTION

In order to reduce error incurred by the transferring of the hybridisation and subsequent addition of the RNase buffer a new procedure was adopted. RNase A and T1 were added at 2 X concentration to 150ul RNase buffer per hybridisation, and then dispensed into 1.5ml Eppendorf tubes. A

TABLE 5: OPTIMUM TEMPERATURE FOR RNA-RNA HYBRID FORMATION.

A minus-sense Gene 8 transcript was hybridised to 20ng of messenger RNA for 16 hours, and RNase digestion performed as described previously. Following TCA precipitation samples were counted and the percentage of RNase resistant radioactivity (as compared to the input radioactivity) was calculated. Table 5 shows that for these RNA duplexes the optimum hybridisation temperature is in the region 37°c to 42°c. 42°c was adopted for routine use.

TEMPERATURE (°C)	% PROBE RNase RESISTANT
15	59%
30	68%
37	87%
42	89%
50	71%

further 150ul of RNase buffer was added to the hybridisation and the 180ul was then mixed with an aliquot of buffer containing the enzymes.

II. TEMPERATURE OF RNASE DIGESTION

To determine the best temperature for complete digestion of single-stranded RNA following hybridisation, a Gene 8 minus sense transcript was hybridised to 20ng of mRNA, and treated with the RNAses at 20, 30, 37 and 45°c. This was followed by TCA precipitation of the residual double stranded RNA. Table 6 indicates that the optimum temperature for RNase digestion is 30° c, since at this temperature the ratio between the sample containing hybridised RNA and that containing probe only is the greatest. Digestion at the higher temperatures decreases the radioactivity in the hybrid samples without a proportional decrease in the background. This could be due to separation of the hybrid strands at the elevated temperature and subsequent digestion of single-stranded areas. At 20°c the RNases appear to function below their optimum, leaving a high background level, with subsequent decrease in contrast. A temperature of 30°c was therefore retained for the RNase digestion.

III. TIME OF RNASE DIGESTION

RNase digestion was varied between 30 minutes and 2 hours, again using hybridisations composed of Gene 8 minus sense transcript and 20ng of mRNA. Results shown in Table 7 indicate that after a 16 hour incubation (preferable to 24 hours, see

TABLE 6: OPTIMUM TEMPERATURE FOR RNase DIGESTION FOLLOWING HYBRIDISATION.

A Gene 8 minus sense transcript was hybridised to 20ng aliquots of Rotavirus messenger RNA. Digestion with RNases was performed at 4 temperatures for 1 hour followed by TCA precipitation. 30° c proved to be the most suitable temperature for digestion.

TEMPERATURE (°C)	20	30	37	45
SAMPLE	RNase	RESISTANT	CPM	
PROBE ONLY	1270	1050	1185	826
PROBE AND mRNA	45,305	44,148	34,651	17,220
FOLD DIFFERENCE	20 X	40 X	30 X	20 X

TABLE 7: EFFECT OF VARIATION OF THE DURATION OF HYBRIDISATION AND RNase DIGESTION ON RNase RESISTANT MATERIAL.

50,000 CPM of a minus sense Gene 8 transcript was hybridised to 20ng aliquots of mRNA or buffer. Hybridisation was allowed to proceed for 16 or 24 hours after which time duplicate samples were digested with RNase A and T1 for 30, 60 and 120 minutes. Following collection of RNase resistant material, the fold excess of sample resistance over background was calculated for each set of data. The conditions producing the best contrast between background and sample were subsequently used.

HYBRIDISATION TIME	16 HOURS		FOLD	24 HOURS		FOLD
RNase DIGESTION TIME	PROBE ONLY	PROBE +mRNA		PROBE ONLY	PROBE +mRNA	
30 MINUTES	2164	42,640	19	7995	36,941	18
60 MINUTES	1679	37,393	22	1474	26,638	18
120 MINUTES	1452	37,189	25	1315	26,187	27

section B) a 2 hour digestion produces the best contrast between hybrid and background resistant CPM, although this increase is not significant over that obtained over a 1 hour digestion. Routinely a 2 hour digestion was used.

IV. CONCENTRATION OF RNASE A AND RNASE T1

The concentrations of RNase A and T1 were reduced from 40ug/ml and 2ug/ml respectively to 20ug/ml and 1 ug/ml respectively without affecting the efficiency of digestion.

V. PROTEINASE K TREATMENT

The amount of Proteinase K added at the end of RNase treatment to terminate the reaction was increased to 100ug. This ensured complete deactivation of the RNases, preventing any further degradation of the hybrids and resulted in a much "cleaner" hybrid as viewed by PAGE.

D. TCA PRECIPITATION

Following RNase digestion, 10ug of tRNA was added to ensure the formation of a good precipitate on the addition of TCA. 330ul of solution is a large volume to apply to filter papers for TCA washing without losing a (variable) proportion of it, and so an alternative approach was adopted; hybrid RNA was precipitated in solution by the addition of 20mM Sodium Pyrophosphate (45ul, 200mM, to decrease background caused by small oligo-nucleotides

binding to the filter) and TCA (45ul, 100%) to a final concentration of 10%. A further 450ul of 20mM Sodium pyrophosphate/10% TCA was added to each tube and samples now 870ul in volume were incubated on ice for 10 minutes, before filtration through glass fibre filters supported on a Millipore manifold. Filters were then washed with 10% TCA/20mM Sodium pyrophosphate *in situ*, rinsed with ethanol and dried by baking at 60° c for a minimum of 1 hour. This final step dried the filters thoroughly, helping to reduce variability in the samples due to quench in the subsequent counting.

E. BACKGROUND CPM INTRINSIC TO THE PROBE RNA

It was important to establish the level of background due to the probe transcript itself. This was achieved by taking samples of a known number of incorporated counts per minute, digesting with RNase under the conditions outlined above and then collecting any remaining precipitable material. Table 8 shows such results obtained using a Gene 8 minus sense transcipt, synthesised at medium (170Ci/mMol) and high (800Ci/mMol) specific activity. The percentage of resistant counts does not remain constant for all 3 samples taken for each specific activity indicating that the residual counts can probably be accounted for in terms of true background, that is non-specific binding of small amounts of ribonucleotides to the filters and not an RNase resistant fraction of the transcript. Since in the final assay conditions, 5×10^5 CPM of the high specific activity probe are used, this background of 0.3% is negligible.

TABLE 8: RNase RESISTANT COUNTS IN "MEDIUM" AND "HIGH" SPECIFIC RNA TRANSCRIPTS.

Transcript samples of known precipitable radioactive content were digested with RNase under the conditions described (2 hours at 30°c), and TCA precipitated to collect intrinsic resistant radioactivity. Percentage resistance was then calculated for each sample.

SPECIFIC ACTIVITY OF TRANSCRIPT	CPM DIGESTED WITH RNase	CPM RNase RESISTANT	% RESISTANT
"MEDIUM" (170Ci/mMOL)	5,000,000	1208	0.02
	500,000	866	0.20
	50,000	755	1.50
"HIGH" (800Ci/mMOL)	5,000,000	3500	0.07
	500,000	1500	0.30
	50,000	1750	3.50

F. INCREASING THE SPECIFIC ACTIVITY OF THE PROBE

Having determined the optimum conditions for the hybridisation assay, it was desirable to improve its sensitivity so that the amount of infected cell RNA needed for each sample could be kept to a minimum, and that very small amounts of viral RNA could be detected. This was achieved simply by removing the cold rCTP from the transcription reaction, which increased the specific activity to that of the isotope itself. This change in transcription conditions was not accompanied by any apparent increase in the level of truncated transcripts, but had the time of transcription been prolonged this would probably have been observed since the concentration of rCTP would become limiting. 5×10^5 CPM of probe was used per hybridisation sample (see section E) representing 330pg of transcript RNA.

DISCUSSION

By making many subtle adjustments to the solution hybridisation assay method it has been possible to reduce the variability between duplicated samples from as much as 100% to between 10 and 20%. In addition it was important to have as large a difference as possible between experimental hybridisation results and the background radioactivity; by adjusting the temperature and time of both the hybridisation and RNase treatment steps this has been achieved as far as possible. Clearly the variability of any assay of this nature is affected by the number

of manipulations it involves, so for this, and for simplicity and rapidity the number of steps has been reduced to a minimum (e.g. a phenol extraction of the hybrid RNA prior to precipitation was not included).

The specific activity of the labelled transcript has been increased as far as possible by using ^{32}-PrCTP labelled at the highest specific activity available (800 Ci/mMol; SP6 Grade). At this level of labelling the RNA is very susceptible to radiolytic degradation and so hybridisations were set up immediately following transcription. After 16 hours at -20°c transcript degradation is already apparent. The specific activity of the transcript could have been increased by using ^{32}-PrATP in addition to the rCTP. However, the use of both isotopes at limiting concentration would have caused many transcripts to be truncated and therefore unsuitable for use in an assay where homogeneity of the probe is essential.

RESULTS: CHAPTER 4.

CHAPTER 4

QUANTITATION OF THE SOLUTION HYBRIDISATION ASSAY

INTRODUCTION

In order to quantitate results using infected cell RNA in this assay, a suitable system of calibration was required so that the amount of RNase resistant radioactivity in a hybridisation could be related to the picogram amount of RNA present in that sample. Each time a set of infected cell samples was analysed, a "calibration curve" was set up alongside it, using the same probe and exactly the same conditions of hybridisation and RNase treatment.

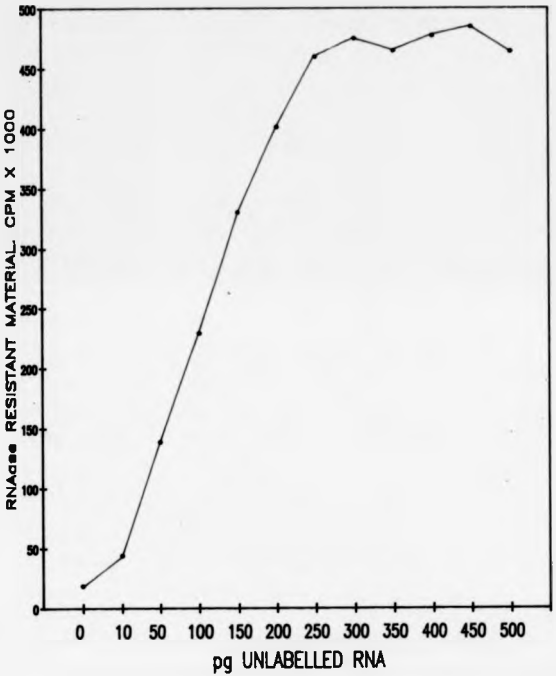
A. THE CALIBRATION CURVE

The calibration curve consisted of a series of hybridisations containing increasing amounts of "cold" RNA complementary to that being used as the probe (usually this was the transcript made using the same template and the other polymerase promotor). Since 500,000 CPM represents 330pg of RNA at the specific activity being used, the calibration curve consisted of duplicate samples containing 0, 10, 50, 100, 150, 200, 250, 300, 400 and 500 pg of the "cold" RNA. On completion of the assay a graph was plotted, using RNase resistant radioactivity on the Y axis and pg of "cold" transcript on the X axis. An example of such a calibration curve is shown in Figure 23.

FIGURE 23: CALIBRATION CURVE FOR GENE 11 PLUS-SENSE RNA DETECTION.

To enable conversion of RNase resistant radioactivity to pg of complementary RNA in the infected cell samples a calibration curve was set up using increasing amounts (10-500 pg) of cold complementary transcript each time a set of infected cell samples was examined. This ensured direct comparability of all sets of results.

FIGURE 23: CALIBRATION CURVE FOR HIGH SPECIFIC ACTIVITY TRANSCRIPT



It is apparent that between 10 and 300pg the amount of RNase resistant radioactivity in a hybridisation is directly proportional to the amount of unlabelled RNA present. Beyond 300pg the probe becomes saturated and the relationship no longer holds. The assay therefore has a 30 fold range of sensitivity; infected cell samples containing between 10 and 300 pg of complementary RNA can be analysed on this curve and it is therefore important that the probe is present in a molar excess for the calibration curve to be effective.

B. ANALYSIS OF INFECTED CELL RNA

All infected cell samples were resuspended in formamide hybridisation buffer and standardised as described in Materials and Methods. As already stated, in order to make the calibration curve functional, all infected cell samples must contain between 10 and 300 pg of Rotavirus RNA complementary to the probe. This can be achieved by adjusting the number of cell equivalents of extracted RNA used in the hybridisations. The amount needed will vary both between genes and with time. Therefore, in order to find a "starting point" for the amounts of cell material to be used a simple hybridisation assay was carried out in which 500,000 CPM of a plus or minus sense Gene 8 transcript was hybridised to increasing amounts of infected cell RNA taken at 8.5 hours post-infection. The results of this assay are presented in Figures 24 and 25.

FIGURE 24: ANALYSIS OF INFECTED CELL RNA ; PLUS-SENSE RNA.

500,000 cpm of a minus-sense Gene 8 specific transcript was hybridised to increasing amounts of infected cell RNA (8.5 hours post-infection). The probe is sub-saturated below 200,000 cell equivalents, and linearity was maintained between 5,000 and 100,000 cell equivalents inclusive. These results indicate the approximate amount of infected cell RNA needed for detection of plus-sense RNA.

MESSENGER RNA DETECTION AT 8.5 HOURS POST-INFECTION USING A GENE 8 PROBE

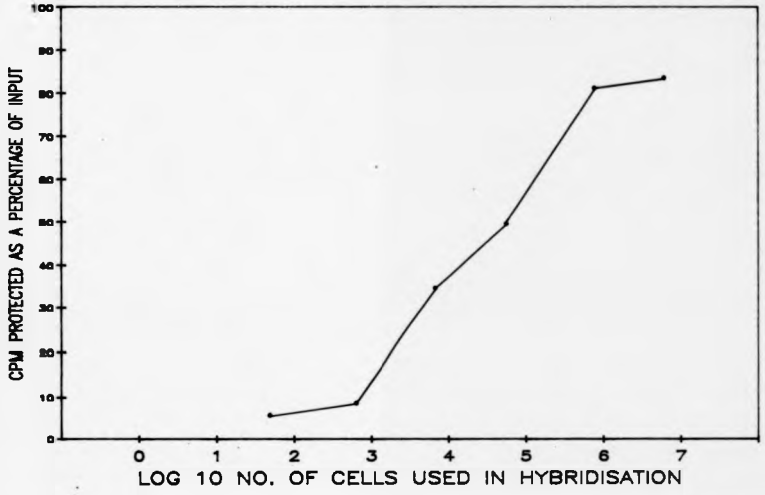
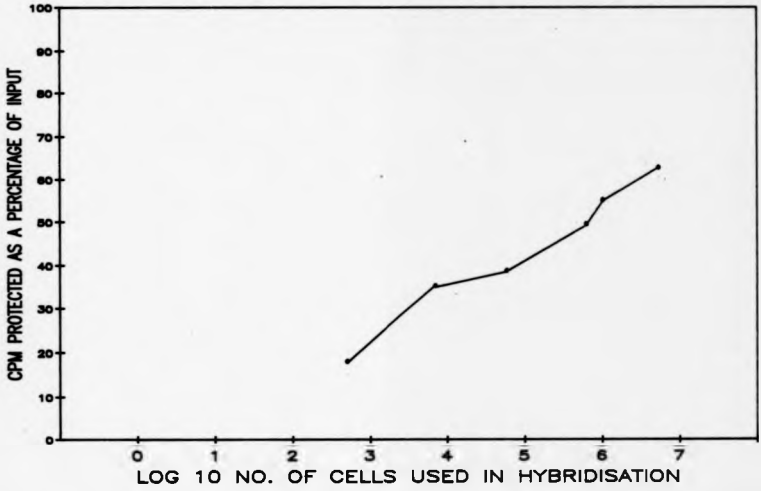


FIGURE 25: ANALYSIS OF INFECTED CELL RNA ; MINUS-SENSE RNA.

500,000 cpm of a plus-sense Gene 8 specific transcript was hybridised to increasing amounts of infected cell RNA (8.5 hours post-infection). The probe remained sub-saturated even when hybridised to 3,000,000 cell equivalents. These results indicate the approximate amount of infected cell RNA needed for detection of minus-sense RNA.

MINUS-SENSE RNA DETECTION AT 8.5 HOURS POST-INFECTION USING A GENE 8 PROBE



Plus-sense RNA was first detectable in 500 cell equivalents, but as only a small percentage of the probe input and not significantly higher than the background level. By the addition of 200,000 cell equivalents the probe was completely saturated, and so in the initial hybridisations with Gene 8 minus-sense probe, between 5000 and 100,000 cell equivalents were used.

Minus-sense RNA was not detected at a level significantly above background until the number of cell equivalents reached 5,000 and the probe was not saturated by the use of as many as 3,000,000 cells. Based on these results between 5000 and 1,000,000 cell equivalents were therefore used in the initial Gene 8 minus-sense assays. It was apparent at this point that at least for Gene 8 there was much more plus-sense RNA than minus-sense RNA at 8.5 hours post-infection, and therefore the detection of minus-sense RNA would require the use of more of the infected cell material. It was assumed that at 8.5 hours post-infection the amount of viral RNA present would be near maximal and so for the earlier samples the amount of RNA used was increased. For the remaining 10 genes, RNA used in the hybridisations was adjusted until 3 sets of data had been obtained in which all experimental samples were on the straight part of the calibration curve.

C. EXAMPLE RESULTS AND CALCULATION

To illustrate the progression from RNase resistant counts in a hybridisation assay to an expression of molecules of RNA per cell, the following example is worked, for Gene 11 plus-sense RNA detection.

Figure 26 is a copy of the calibration curve of Figure 23, with the positions of the infected cell samples indicated. Table 8 shows numbered stages in the calculation in Figure 27, which was used for each set of data generated.

D. SENSITIVITY OF THE HYBRIDISATION ASSAY.

The limit of detection of the hybridisation assay is taken to be that number of molecules per cell corresponding to 10pg of RNA (since this is the smallest amount of RNA used in the calibration curve, and is usually only approximately 2.5 fold higher than the background level) and depends on the highest number of cell equivalents used in the assay (this was always used for the uninfected samples and compared well with the background level of resistant counts) and the proportion of the Gene represented by the hybrid RNA. In the results presented in C) the limit of detection was 34 molecules of RNA per cell.

DISCUSSION

The results presented in Chapter 4 indicate that Rotavirus RNA in infected cells can be detected sensitively and accurately by the Solution Hybridisation method and even in the early stages of infection all RNAs are present at levels within

FIGURE 26: EXAMPLE RESULTS: GENE 11 PLUS-SENSE RNA DETECTION.

The calibration curve shown in Figure 23 is represented showing the positions of the infected cell samples. Using the linear portion of the curve it is possible to convert RNase resistant radioactivity to pg of specific RNA present in the samples.

FIGURE 26: EXAMPLE RESULTS. (GENE 11)
PLUS-SENSE RNA DETECTION (mRNA).

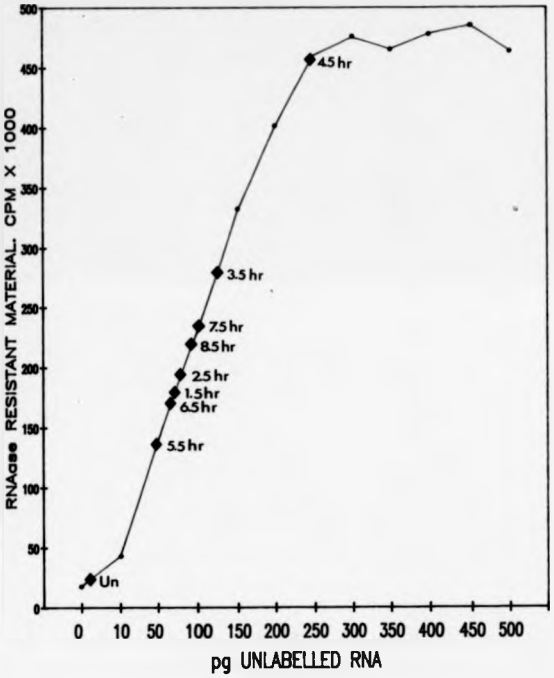


TABLE 9 AND FIGURE 27. CONVERSION OF RNase RESISTANT RADIOACTIVITY TO MOLECULES OF RNA PER CELL FOR GENE 11 PLUS-SENSE RNA.

Figure 27 shows the calculation applied to each set of data and Table 9 shows actual results obtained at each stage of the calculation. The numbers in brackets indicate stages in the calculation in Figure 27 and correspond to the data presented in Table 9.

The additional information used in calculation but not shown in Table 9 is:

$$\frac{\text{Gene size (3)}}{\text{Hybrid size (4)}} = 1.3$$

Approximate molecular weight of the Gene 11 c-DNA (5) = 230,000

CALCULATION TO CONVERT RNase RESISTANT RADIOACTIVITY IN A HYBRIDISATION ASSAY TO MOLECULES OF RNA PER CELL.

READ pg RNA DETECTED FROM CALIBRATION CURVE:

$$\frac{\text{pg RNA DETECTED (1)}}{\text{CELLS IN HYBRIDISATION (2)}} = \text{pg RNA PER CELL}$$

$$\text{pg RNA PER CELL} \times \frac{\text{GENE SIZE IN BASE PAIRS (3)}}{\text{HYBRID SIZE IN BASE PAIRS (4)}} = \text{CORRECTED pg RNA PER CELL}$$

$$\frac{\text{CORRECTED pg RNA PER CELL}}{\text{MOLECULAR WEIGHT OF GENE (5)}} = \text{MOLES PER CELL (6)}$$

$$\text{MOLES PER CELL (6)} \times \text{AVOGADRO'S NUMBER} = \text{MOLECULES PER CELL (7)}$$

TIME, HOURS P-I	CPM PROTECTED	PgRNA (1)	NO. OF CELLS IN ASSAY (2)	MOLES/CELL (6)	MOLECULES /CELL (7)
UNINFECTED	22,899	<10	1×10^{6}	5.6×10^{-23}	<34
1.5	174,960	71	1×10^{6}	3.9×10^{-22}	240
2.5	189,025	79	5×10^{5}	9.0×10^{-22}	545
3.5	256,069	111	5×10^{4}	1.2×10^{-20}	7,490
4.5	456,550	245	5×10^{4}	2.7×10^{-20}	16,680
5.5	138,020	53	1×10^{4}	3.0×10^{-20}	18,042
6.5	172,640	62	1×10^{4}	3.5×10^{-20}	21,100
7.5	233,180	102	1×10^{4}	5.7×10^{-20}	34,725
8.5	219,420	96	1×10^{4}	5.4×10^{-20}	32,680

the range of the assay (see Chapters 5 and 6). Adjustment of the infected cell samples to the linear part of the calibration curve is achieved empirically, using experience as a guide to an appropriate starting point. Figures 24 and 25 indicate that there is between 10 and 20 times more Gene 8 plus-sense RNA than Gene 8 minus-sense RNA in infected cells at 8.5 hours post-infection. Since 50% probe saturation is achieved at 50,000 cell equivalents in the former and between 500,000 and 1,000,000 in the latter.

COMPARISON OF SOLUTION HYBRIDISATION ASSAY WITH A DOT-BLOT BASED ASSAY.

To enable comparison of the new solution hybridisation method with a Dot-blot method two identical filters were prepared loaded with a range of cold Gene 7 specific transcripts (of both strands) Rotavirus mRNA and Genomic (ds)RNA, and also tRNA and Reovirus dsRNA (negative controls) and infected and uninfected cell samples as shown in Figures 28 and 29. These filters were hybridised to Gene 7 specific transcripts, Figure 28 shows the filter probed with a minus-sense transcript while Figure 29 shows a similar filter hybridised to a plus-sense transcript. It is evident that transcripts hybridise only to the cold transcript of the opposite orientation or to Rotavirus RNA, there is no background hybridisation to a transcript of the same sense (and in the case of the plus-sense transcript to mRNA) or to the unrelated RNAs. Background hybridisation to the uninfected cell samples is negligible when compared to the signals produced by infected cells.

FIGURES 28 AND 29: DOT-BLOT HYBRIDISATION WITH THE HIGH SPECIFIC ACTIVITY TRANSCRIPT USED IN SOLUTION HYBRIDISATION.

T7 and SP6 transcripts of a Gene 7 c-DNA subclone (in pGEM1) were hybridised to homologous RNAs, un-related RNAs and infected cell material, in order to show specificity of the transcripts and their sensivity in Dot-Blot assays.

Nylon Membranes were loaded as follows:

Row 1A: (Figure 29 only) MINUS-SENSE TRANSCRIPT: E)10ng, F)1ng, G)500pg, H)10pg, I)1pg, J)100fg.

Row 1: PLUS-SENSE TRANSCRIPT: E)10ng, F)1ng, G)500pg, H)10pg, I)1pg, J)100fg.

Row 2: ROTAVIRUS mRNA (6% of this is Gene 7 specific sequence): A) 1ug, B)500ng, C)100ng, D)50ng, E)10ng, F)1ng, G)500pg, H)10pg, I)1pg, J)100fg.

Row 3: ROTAVIRUS GENOMIC RNA (3% of this is Gene 7 specific sequence): A) 1ug, B)500ng, C)100ng, D)50ng, E)10ng, F)1ng, G)500pg, H)10pg, I)1pg, J)100fg.

Row 4: TRANSFER RNA (from Torula yeast): A) 1ug, B)500ng, C)100ng, D)50ng, E)10ng, F)1ng, G)500pg, H)10pg, I)1pg, J)100fg.

Row 5: REOVIRUS TYPE 2 GENOMIC RNA: A) 1ug, B)500ng, C)100ng, D)50ng, E)10ng, F)1ng, G)500pg, H)10pg, I)1pg, J)100fg.

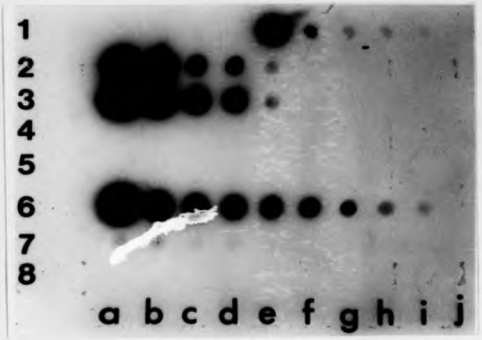
Row 6: INFECTED CELL RNA (6 hours post-infection): A) 3,000,000 B)2,000,000 C)1,000,000 D)500,000 E)100,000 F)50,000 G)10,000 H)5,000 I)1,000 J)100 cell equivalents of RNA.

Row 7: UNINFECTED CELL RNA: A) 3,000,000 B)2,000,000 C)1,000,000 D)500,000 E)100,000 F)50,000 G)10,000 H)5,000 I)1,000 J)100 cell equivalents of RNA.

Row 8: (Figure 29 only) As Row 1A.

FIGURE 28: HYBRIDISATION TO A MINUS-SENSE GENE 7 HIGH SPECIFIC ACTIVITY TRANSCRIPT (SP6 TRANSCRIPT OF CLONE 17).

FIGURE 29: HYBRIDISATION TO A PLUS-SENSE GENE 7 HIGH SPECIFIC ACTIVITY TRANSCRIPT (T7 TRANSCRIPT OF CLONE 17).


1
2
3
4
5
6
7
8
a b c d e f g h i j

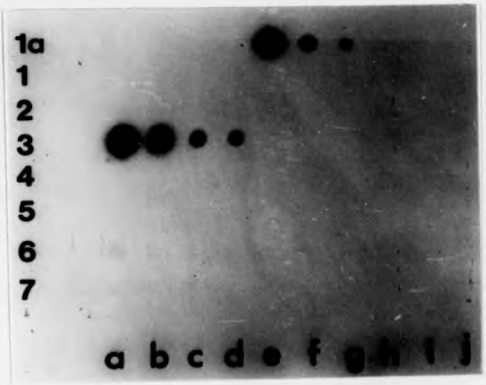

1a
1
2
3
4
5
6
7
a b c d f

Figure 28 shows that plus-sense transcript is detectable by autoradiography at 10pg. However at this level the signal is very poor (autoradiography was for 5-7 days) and comparable in strength to the background of the uninfected samples. A clear positive detection by this method requires 1ng of complementary RNA and it is doubtful whether there would be a linear relationship between RNA bound to the filter and the level of hybridisation obtained below that level. It has already been illustrated that the solution hybridisation method can be used for calibration to as little as 10pg of RNA. The results shown in Figure 28 and 29 took up to 7 days to obtain, due to time needed for autoradiography. During this time the ^{32}-P will have lost approximately one quarter if its activity and therefore reduced numbers of CPM will be obtained on counting the excised Dots. Results using the solution Hybridisation may be obtained within two days. In spite of the same total amount of labelled transcript being used in both assays the level of recovery of radioactivity was much higher in the solution hybridisation. For example for 500pg of RNA there were approximately 400,000 CPM RNase resistant by solution hybridisation whereas the excised dot bearing 500pg of RNA contained only approximately 1000 CPM. This would appear to indicate that in solution hybridisation more of the complementary RNA was available for hybridisation.

Comparison of Figures 28 and 29 confirms the earlier observation that there is very much more plus-sense RNA than minus-sense RNA in the infected cells (these are samples taken at 6.5 hours post-infection). Gene 7 plus-sense RNA can be clearly detected (at a level significantly above background) in 10,000 cells by Dot-Blotting. By solution hybridisation only

5,000 cells are required to measure this level of RNA, thus reducing the amount of infected cell material needed for the assay (an important point when analysis on both strands of all 11 genes needs to be carried out on the same infected cell stocks). Figure 29 indicates that by Dot-Blotting Gene 7 minus sense RNA cannot be detected in as many as 3,000,000 cells, while only 500,000 cells were needed for that same time point by solution hybridisation.

RESULTS: CHAPTER 5.

CHAPTER 5

ACCUMULATION OF ROTAVIRUS PLUS-SENSE RNA DURING THE REPLICATION CYCLE AND DEMONSTRATION OF TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL IN GENE EXPRESSION.

INTRODUCTION

The modifications to the basic solution hybridisation method described in Chapter 3 reduced the variation between duplicate samples to between 10 and 20%, while the calibration curve described in Chapter 4 allowed the conversion of RNase resistant radioactivity in a hybridisation assay to molecules of RNA per cell. In order to determine the levels of messenger and minus-sense RNA for each of the eleven genes at each time post-infection (upto and including 8.5 hours PI at hourly intervals) in a reproducible fashion it was important that all of the hybridisations were carried out on the same infected cell RNA stock. To accommodate this, 2 X 850cm rolling culture bottles of cells (150 million cells) were prepared for each time point (see Methods Section 2). The procedure followed for extracting RNA from tissue culture cells involved several phenol extractions, and so it was likely that recovery of RNA would vary between samples. To ensure that the hybridisation results obtained throughout the infection would be directly comparable, all cells were "pre-labelled" with ^{3}-H Uridine prior to infection (see Methods Section 2). The Uridine which would become incorporated into

cellular RNA could then be used as a means of standardising the RNA stocks, by measuring TCA precipitable ^{3}-H. The volumes of the RNA stocks were adjusted with hybridisation buffer so that the cellular RNA content per unit volume was constant between all samples.

Determination of messenger RNA accumulation was then carried out for all eleven genes. The results obtained are summarised in graphical form in this Chapter and numerical data is appended in Table 15. The infected cell protein profile shown in Figure 31 suggests that viral polypeptides such as VP6 and VP8 may be produced in larger quantities than the remaining polypeptides, in particular VP9 and VP11. Because of the labelling procedure used (Methods Section 7: Methionine is an uncommon amino acid) it is possible that this apparent difference is an artefact produced by the methodology, and so we decided to quantitate the relative molar amounts of the Rotavirus polypeptides produced at 6.5 hours post-infection, when protein synthesis is readily monitored by radio-labelling. The results of the protein quantitation are presented in this Chapter, enabling the direct comparison of transcription and translation of the eleven Rotavirus genes.

RESULTS

A. ACCUMULATION OF MESSENGER RNA

A compilation of data for plus-sense RNA accumulation is shown in Figure 30, parts A to K in which molecules of RNA per cell is plotted versus time post-infection for each gene in turn.

It should be noted that due to the computer software used for plotting these figures (Ashton-Tate Chartmaster) it was not possible to standardise the Y axis and so the Y axis scales should be carefully observed when interpreting this data.

Differences in messenger-RNA accumulation of the 11 Rotavirus genes were apparent at two levels:

1. TEMPORAL VARIATION

Accumulation of mRNA of all 11 genes was apparent by 2.5 hours PI, and continued to increase until at least 7.5 hours PI. On the basis of the mRNA accumulation data presented in Figure 30, the genes could be divided into three broad categories; i) Genes for which mRNA accumulation proceeded at a more or less steady rate throughout infection eg. Genes 1, 2, 6, 8 and 11. ii) A gene for which mRNA accumulated more rapidly earlier in infection and then reached a plateau later in infection; Gene 7, and iii) Genes for which mRNA accumulated relatively slowly early in infection but more rapidly later in infection, eg Genes 3,4,5,9 and 10.

There did not appear to be any distinct division or switch between "early" and "late" messenger RNAs, during the Rotavirus infection studied. However it was noticeable that mRNA of several genes eg. 1,2 and 7 accumulated earlier than the others, but because of the poor inhibition of host cell protein synthesis, it was not possible to measure relative levels of the viral polypeptides at these early times (3.5-4.5 hours) PI. Therefore it could not be concluded that early accumulation of mRNA meant early synthesis of the corresponding polypeptides.

FIGURE 30 PARTS A-K: ACCUMULATION OF MESSENGER RNA.

The accumulation of plus-sense (messenger) RNA was measured for each of the 11 Rotavirus genes at hourly intervals (as indicated) throughout an 8.5 hour simultaneous infection as previously described. The results obtained for genes 1 to 11 are labelled A through K respectively. It should be noted that due to the software used for plotting these graphs it was not possible to standardise the Y axis scales, and so attention should be paid to the Y axis scale on each part of Figure 30.

FIGURE 30. PART A: ACCUMULATION OF GENE 1 PLUS-SENSE (MESSENGER) RNA.

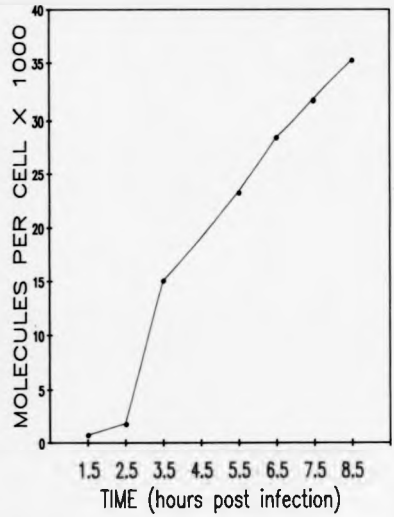


FIGURE 30. PART B: ACCUMULATION OF GENE 2 PLUS-SENSE (MESSENGER) RNA.

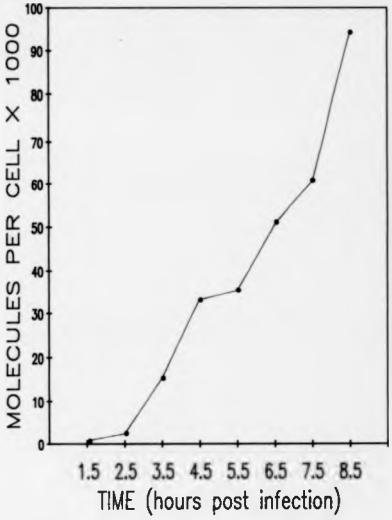


FIGURE 30. PART C: ACCUMULATION OF GENE 3 PLUS-SENSE (MESSENGER) RNA.

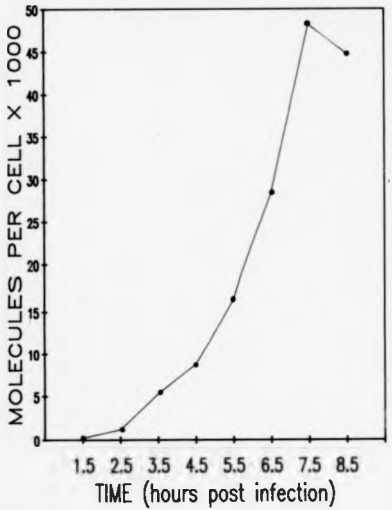


FIGURE 30. PART D: ACCUMULATION OF GENE 4 PLUS-SENSE (MESSENGER) RNA.

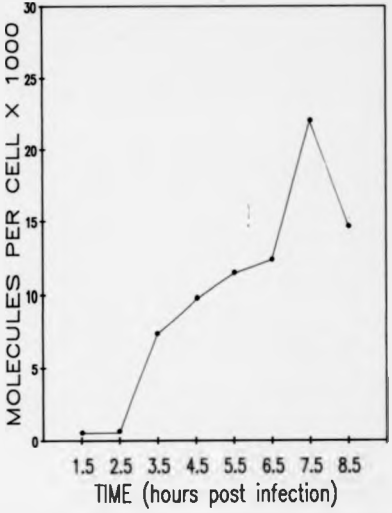


FIGURE 30. PART E: ACCUMULATION OF GENE 5 PLUS-SENSE (MESSENGER) RNA.

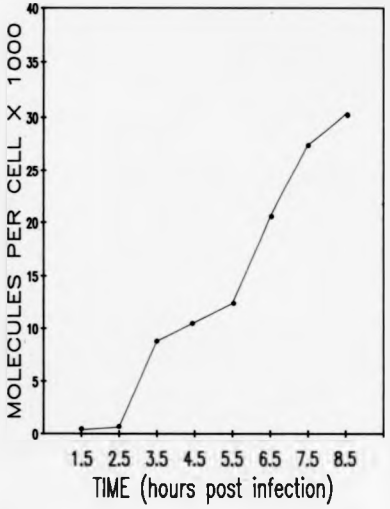


FIGURE 30. PART F: ACCUMULATION OF GENE 6 PLUS-SENSE (MESSENGER) RNA.

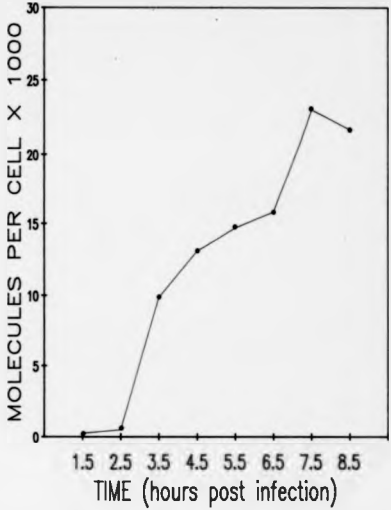


FIGURE 30. PART G: ACCUMULATION OF GENE 7 PLUS-SENSE (MESSENGER) RNA.

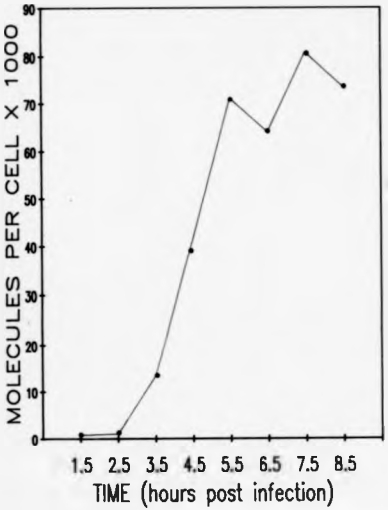


FIGURE 30. PART H: ACCUMULATION OF GENE 8 PLUS-SENSE (MESSENGER) RNA.

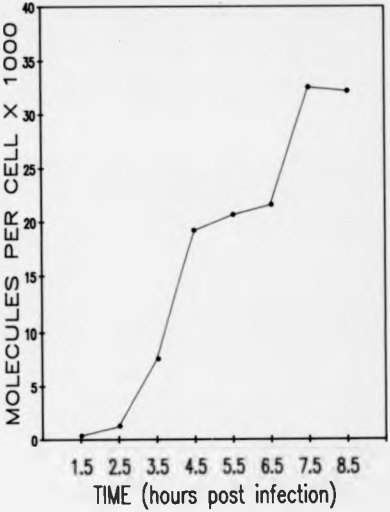


FIGURE 30. PART I: ACCUMULATION OF GENE 9 PLUS-SENSE (MESSENGER) RNA.

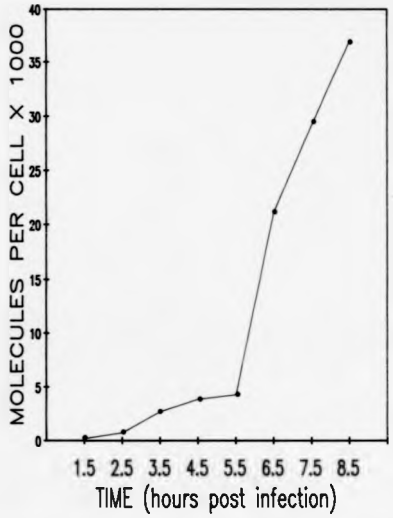


FIGURE 30. PART J: ACCUMULATION OF GENE 10 PLUS-SENSE (MESSENGER) RNA.

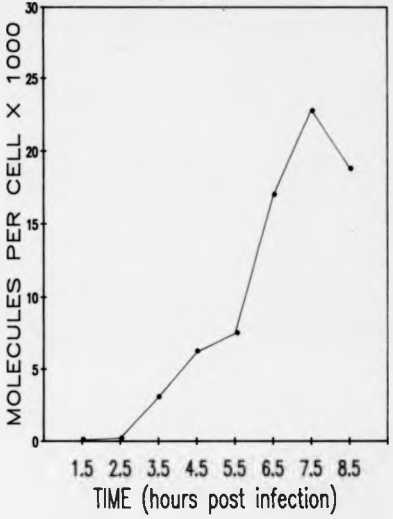


FIGURE 30. PART K: ACCUMULATION OF GENE 11 PLUS-SENSE (MESSENGER) RNA.

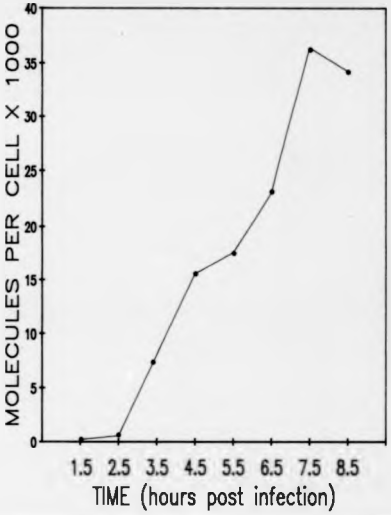
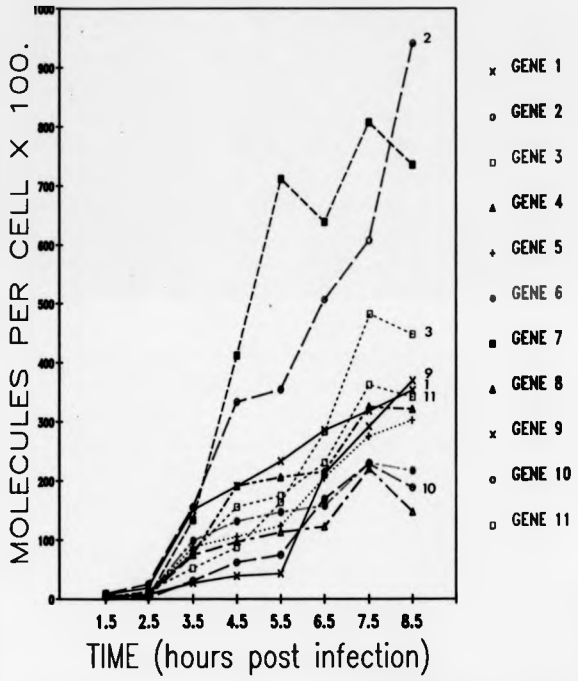


FIGURE 30 SUMMARY: ACCUMULATION OF PLUS SENSE (MESSENGER) RNA.



2. QUANTITATIVE VARIATION

Quantitative analysis of mRNA accumulation upto and including 7.5 hours PI allowed the Rotavirus genes to be divided into 4 broad categories; 1) mRNA of Genes 2 and 7 were accumulated to the highest level (73-84,000 molecules/cell) 2) mRNA of Gene 3 was accumulated to 45,000 molecules/cell, 3) mRNA of Genes 1, 5, 8, 9 and 11 were accumulated to 30-36,000 molecules/cell and 4) mRNA of genes 4, 6 and 10 were accumulated to the lowest levels(14-21,000 molecules/cell). It was possible that the levels of mRNA accumulated during infection would be reflected in the quantities of viral polypeptides produced, and their respective functions. Since the coding assignments of all of the Rotavirus genes are known (McCrae and McCorquodale 1982a and Figure 2) and the functions of some of these have been determined, it was possible to quantitate the production of the viral polypeptides during a 15 minute period and compare the results with the quantities of messenger RNA accumulated as illustrated in Figure 30 parts A to K. By measuring both mRNA and polypeptide production during infection it was possible to illustrate the occurrence of both transcriptional and translational control.

B. POLYPEPTIDE PRODUCTION DURING ROTAVIRUS REPLICATION

The production of the Rotavirus proteins during the 15 minute period was measured at 6.5 hours PI as described in Methods Section 7. Following the pulse-chase labelling procedure, viral polypeptides were fractionated on 5-11% polyacrylamide gels and treated with PPO for fluorography. The resulting autoradiograph (for an example see Figure 31) was used as a template to excise parts of the gel containing the labelled polypeptides, and ^{35}-S counted in scintillation fluid. To convert ^{35}-S CPM per gel slice into a quantitative expression of protein synthesis, the following steps were carried out: Firstly background radioactivity was deducted from each gel slice (measured by taking a slice from an area without protein bands). Secondly, because methionine is a relatively uncommon amino acid, results were adjusted according to the methionine content of each polypeptide. The percentage of methionine residues in each protein was determined from published sequence data using the Staden "Analyseq" program. The percentage methionine contents used in the calculations are shown in Table 10. For the 3 genes not yet sequenced (genes 1,2 and 3), the mean of the 8 proteins shown was used in calculation. When sequence for these genes becomes available the data can be recalculated appropriately. As VP12 has been shown to be the unglycosylated protein-precursor of VP10 (McCrae and Faulkner-Valle 1981) the gel slices for these two proteins were counted together. To represent relative molar amounts of protein all data were then adjusted according to the approximate molecular weight of the final protein product (from McCrae and Faulkner-Valle 1981) and presented as a relative value to VP11. Data from 5 separate protein profiles was combined and the mean presented in Table 11. Of the 11 final protein products,

FIGURE 31: ROTAVIRUS SPECIFIC POLYPEPTIDES FRACTIONATED BY P.A.G.E.

Infected cells were pulse labelled for 15 minutes at 6.5 hours post-infection with 35-S methionine. Cells were harvested into 50mM Tris-HCl pH8.0 and fractionated on 5-11% polyacrylamide gels. For pulse-chase analysis cells were pulse-labelled as above, at the end of the pulse period the isotope was removed and replaced with medium containing 100 times the normal amount of methionine. Cells were harvested as described after 2 hours. Gels were treated with PPO for fluorography, and protein bands excised using the autoradiograph as a template and counted in liquid scintillant (see Table 11).

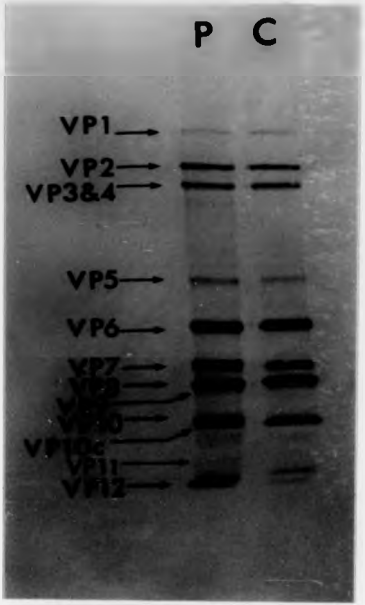

P
C
VP1
VP2
VP3&4
VP5
VP6
VP7
VP8
VP9
VP10
VP10a
VP11
VP12

TABLE 10: METHIONINE CONTENT OF THE ROTAVIRUS PROTEINS.

Methionine content of 8 of the 11 Rotavirus encoded polypeptides was obtained from published sequence data using the Staden "Analyseq" software package. For VP1, 2 and 3 the mean of the 8 values shown in Table 10 was used in calculation. When sequence data for these genes becomes available the results could be recalculated accordingly.

PROTEIN	PROTEIN LENGTH (AMINO ACIDS)	NUMBER OF METHIONINES	PERCENTAGE METHIONINES
VP4	755	38	5.0
VP5	491	13	2.6
VP6	397	13	3.3
VP7	326	8	2.5
VP8	317	15	4.7
VP9	313	21	6.7
VP10	175	3	1.7
VP11	198	10	5.0

9 could be resolved completely, VP3 and VP4 proved difficult to resolve and so data for these is combined.

From Table 11 it is evident that the Rotavirus proteins were not produced in equal amounts at 6.5 hours PI. Two of the virus specific proteins were produced in a very large molar excess over the remaining nine, these were VP6 and VP10. VP6 is the major constituent of the inner capsid shell of the virus particle, and VP10 is a non-structural glycoprotein (see Introduction 1iiC Section). VP8 which has not yet been unequivocably designated structural or non-structural was also produced in relatively large quantities although at only half the molar amount of VP6. The predominance of VP8 in protein profiles appeared to be partly due to its high methionine content (4.7%) compared to the other viral polypeptides. VP9, the other non-structural protein and VP7, the neutralisation antigen were produced in a four-fold excess over VP11, while the remaining proteins, which with the exception of VP11 (whose location is not known) are constituents of the inner or outer virion shell were produced in very small molar quantities. VP1 was produced at only 1/260th the rate of production of VP10 at this time post-infection.

The polypeptides synthesized in the pulse were followed into a chase period and the results treated as outlined above. Since changes may be occurring in all proteins it is difficult to analyse the data objectively, although it is possible to make several general observations. Since all of the polypeptides show a decreased relative molar amount to VP11 it would seem likely that VP11 has shown an increase. This could be indicative of a precursor-product relationship, but such a

TABLE 11: ROTAVIRUS PROTEIN PRODUCTION AT 6.5 HOURS POST-INFECTION (RELATIVE TO VP11).

Infected cells were pulse labelled with 35-S methionine, and labelled polypeptides fractionated by P.A.G.E. Following fluorography the level of each polypeptide was calculated as described in the text and is presented as a relative to VP11 for both pulse and 2 hour chase samples.

PROTEIN	RELATIVE MOLAR AMOUNTS	
	PULSE	CHASE
VP1	0.15 X +/- 0.05	0.08 X +/- 0.05
VP2	1.32 X +/- 0.14	0.54 X +/- 0.11
VP3/4	1.27 X +/- 0.12	0.54 X +/- 0.10
VP5	1.57 X +/- 0.18	0.47 X +/- 0.09
VP6	25.02 X +/- 2.52	5.60 X +/- 0.73
VP7	3.48 X +/- 0.64	2.18 X +/- 0.61
VP8	10.68 X +/- 2.35	3.57 X +/- 0.87
VP9	4.43 X +/- 1.84	0.91 X +/- 0.62
VP10	38.65 X +/- 3.43	14.13 X +/- 2.19
VP11	1 X +/- 0.07	1 X +/- 0.08

relationship has not been demonstrated for VP11. However, since VP11 is produced in very small amounts and is always a very feint band in electrophoresis/autoradiography the possible existence of a precursor should not be dismissed. It should be noted that in the case of VP7 there is a mobility shift between the pulse and the chase periods; in the pulse samples VP7 is measured, whilst in the chase, the final product VP7c is measured. Although the precursor-product relationship between VP10, VP10c and VP12 was not examined quantitatively here it can be seen in Figure 28 that bands VP12 and VP10 are greatly reduced in the chase leaving predominantly the final product VP10c.

C. THE ROLE OF TRANSLATIONAL CONTROL IN ROTAVIRUS REPLICATION

It has already been illustrated (Section B) that production of the 11 final protein products during Rotavirus replication is unequal, and therefore some form of control mechanism is clearly in operation. This control could take place at either one of two levels; Transcription and/or translation. It has already been shown (Section A) that transcriptional control occurs during replication, and it is now important to determine whether this is entirely responsible for the different levels of protein produced, or if translational control also plays a part.

The relative levels of mRNA and protein production for each gene-product pair is expressed relative to gene 11 in Table 12. Examination of these results indicates that the level of mRNA is not directly reflected in the relative molar quantity of protein produced. For example, gene 10 mRNA accumulates to one

TABLE 12: TRANSLATION AND TRANSCRIPTION OF THE 11 ROTAVIRUS GENE PRODUCTS.

Results obtained for mRNA (Figure 30 parts A to K) and polypeptide production (Table 11) are presented to illustrate transcriptional and translational control during Rotavirus replication in tissue culture. Since the absolute amounts of protein could not be measured in this procedure it was not possible to calculate molecules of protein per cell (the level and effects of isotopic dilution were unknown) and therefore the data shown in Table 12 is a relative assessment only.

PROTEIN/ GENE	RELATIVE PRODUCTION OF PROTEINS (to VP11)	RELATIVE ACCUMULATION OF mRNA (to VP11)	TRANSLATION/TRANS-CRIPTION FREQUENCY
VP1/SP1	0.15	1.3	0.1
VP2/SP2	1.32	2.3	0.5
VP3/SP3	-	1.3	-
	1.27		0.6
VP4/SP4	-	0.6	-
VP5/SP5	1.57	0.9	0.6
VP6/SP6	25.02	0.7	36.6
VP7/SP8	3.48	0.9	3.9
VP8/SP7	10.68	2.8	3.8
VP9/SP9	4.43	0.9	4.9
VP10/SP10	38.65	0.7	54.3
VP11/SP11	1	1	1

third the level of Gene 7 mRNA (at 6.5 hours post-infection) while Vp10 is produced in approximately four times the molar quantity of Vp8, suggesting that the Gene 10 mRNA is translated at 12 times the efficiency of Gene 7 mRNA. Similar comparisons can be drawn between other pairs of genes. It appears that the 5 polypeptides other than Vp6 thought to form the inner capsid shell (although the location of Vp5 has not yet been precisely defined) are produced in relatively small quantities due to a poor relative translational efficiency since the mRNAs for these proteins accumulate to similar or higher quantities than some of the other mRNA species. By contrast proteins produced in large amounts eg. Vp6 and Vp10 have a high translation/transcription frequency while Vp8 appears to depend more on mRNA production than translation to achieve its relatively high levels. It can therefore be concluded that both transcriptional and translational control interact to regulate protein production in Rotavirus infection.

DISCUSSION

The levels of mRNA and protein produced during infection can, in some cases, be speculatively related to the function of the final gene products. Gene 7 mRNA, encoding VP8 appeared to be produced early in infection only. Vp8, thought by some groups to be non-structural (Estes et al 1983) but by McCrae and Faulkner-Valle (1981) to be a structural protein in the UKtc strain, has been shown to have RNA binding properties (McCrae and Baybutt personal communication). The rapid accumulation of gene 7 mRNA early in infection (upto 5.5 hours) could indicate that VP8 is more important at this stage of replication, and together with

the RNA binding properties of this protein suggests that it may play a role in the early stages of morphogenesis. For example, on the assumption that Rotavirus morphogenesis proceeds via the same mechanism as Reovirus morphogenesis (Introduction Section 2 I) it is possible that VP8 has a role in the assembly of sets of mRNA molecules. Similarly the more rapid accumulation of mRNA for genes 9 and 10 (encoding the non-structural proteins VP8 and VP10) could suggest a regulatory role later in morphogenesis.

Genes 1, 2 and 3 are candidates for the genes encoding the transcriptase and replicase activities. mRNA for Gene 3 accumulated at a higher rate later in infection whilst those for Genes 1 and 2 accumulated steadily throughout. Recent evidence from Gombold and Ramig (1987) indicates that gene 1 may encode the transcriptase and gene 2 the replicase activities: A ts mutant of SA11 Rotavirus with a ssRNA negative-dsRNA negative phenotype mapped to VP1 in SA11 ts mutant X RRV ts mutant crosses, while a second mutant with a ds-RNA negative phenotype (and able to synthesise ssRNA) mapped to VP2 in similar crosses. These authors failed to find a selectable marker located on segment 3 in their collection of ts mutants. These RNA negative phenotypes are consistent with the location of VP1 and VP2 in viral cores (Bican et al 1982) and the recent demonstration of intracellular ssRNA and dsRNA synthesis in particles containing VP1 and VP2 (Helmberger-Jones and Patton 1986). Gene 2 was accumulated at twice the quantity of Gene 1, while the protein product VP2 was produced in 9 times the molar quantity of VP1 at 6.5 hours post infection. These observations suggest that VP2 is required in much larger quantities than VP1 for its enzymic activity, and when the fact that on average there is 40 times more mRNA produced per

cell than minus-sense RNA (see Chapters 5 and 6) is also considered it would appear that the replicase (according to the coding assignments of Gombold and Ramig (1987)) is either a much less active enzyme than the transcriptase, or that many sub-units of VP2 are required to produce an enzymic site.

Table 12 illustrates that gene expression in the UKtc Rotavirus system is subject to both transcriptional and translational control, genes such as 6 and 10 appear to depend predominantly on a high translational frequency to achieve their high levels of expression, while others eg. genes 1 and 7 appear to regulate protein production mainly by the levels of mRNA produced. How the translational control is exerted is unknown. Recently there have been attempts to identify sequences in Reovirus mRNA molecules which may determine frequency of translation (Joklik personal communication), these sequences which are thought to be located in the 5' untranslated region of the mRNAs may also be present in the Rotavirus messenger RNAs. As progress is made with the work in Reovirus it may be possible to apply any findings to the Rotavirus mRNA sequences and to the data presented in this Chapter.

In 1981 McCrae and Faulkner-Valle acknowledged that temporal control of gene expression played a part in replication. They observed from polyacrylamide gel analysis that there were three classes of polypeptides; i) Those such as VP9 which reached their maximum rate of synthesis by 2 hours post-infection, ii) Others such as VP1 which did not reach their maximum rate until 4 hours post-infection, and iii) Those like VP6 whose rate of synthesis appeared to increase throughout infection. Reference to Figure 30 A-K indicates that for all genes accumulation of mRNA

continues until at least 7.5 hours post-infection and so it appears that translational control must be operative otherwise one would expect rate of synthesis for all proteins to continue to increase throughout infection,ie.rate or frequency of translation must decrease with time in the case of the first two groups of proteins referred to above. Alternatively mRNA may be structurally altered such that is cannot be translated, but is still able to hybridise to the RNA probes used in the assay. This inability of the assay to distinguish between functional and non-functional mRNA molecules needs to be taken into account when interpreting the data. Also important in the interpretation of the data in this Chapter and in Chapter 6 is that the assay does not take into account decay of RNA molecules. All data presented is cumulative in nature. By 8.5 hours post-infection several of the mRNAs start to decline which could indicate either that their intrinsic decay rate has exceeded the rate of synthesis or that they are being destroyed due to the release of nucleases from the dying cells towards the end of the infectious cycle. This last point will be reconsidered (see General Discussion).

RESULTS: CHAPTER 6.

CHAPTER 6

ACCUMULATION OF MINUS-SENSE RNA AND THE PRODUCTION OF PROGENY VIRUS PARTICLES.

INTRODUCTION

The accumulation of minus-sense RNA was followed in the same way as that of plus-sense RNA. The observation that there are equimolar amounts of each of the 11 genes in infectious Rotavirus particles suggested that there would also be equimolar amounts of the minus-sense strands in infected cells. Any deviation from an equimolar situation would suggest the generation of defective particles, since it is thought that minus sense RNA is only synthesised in nascent virus particles (by analogy to Reovirus). During the course of the infection aliquots of the infected cells collected at each time post-infection were reserved for titration of infectivity. The results of the titration can be compared with the Particle:PFU ratio calculated for the last (8.5 hour PI) sample, and the results for the minus-sense strands of the 11 genes as obtained by Solution Hybridisation.

RESULTS

A. ACCUMULATION OF MINUS-SENSE RNA

FIGURE 32 PARTS A-K: ACCUMULATION OF MINUS-SENSE RNA.

The accumulation of minus-sense RNA was measured for each of the 11 Rotavirus genes at hourly intervals (as indicated) throughout an 8.5 hour simultaneous infection as previously described. The results obtained for genes 1 to 11 are labelled A through K respectively. It should be noted that due to the software used for plotting these graphs it was not possible to standardise the Y axis scales, and so attention should be paid to the Y axis scale on each part of Figure 32. The Y axes for Figure 32 are 100 times smaller than those seen is Figure 30, illustrating the large differences between accumulation of the two complementary strands.

FIGURE 32. PART A: ACCUMULATION OF GENE 1 MINUS-SENSE (COMPLEMENTARY) RNA.

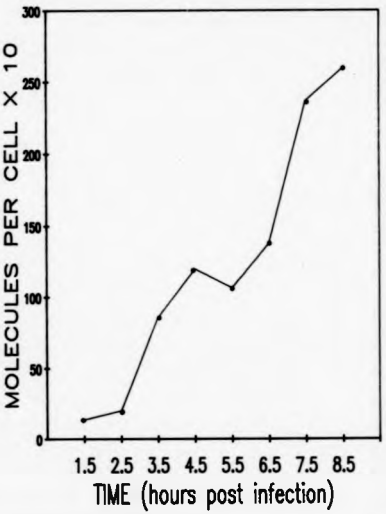


FIGURE 32. PART B: ACCUMULATION OF GENE 2 MINUS-SENSE (COMPLEMENTARY) RNA.

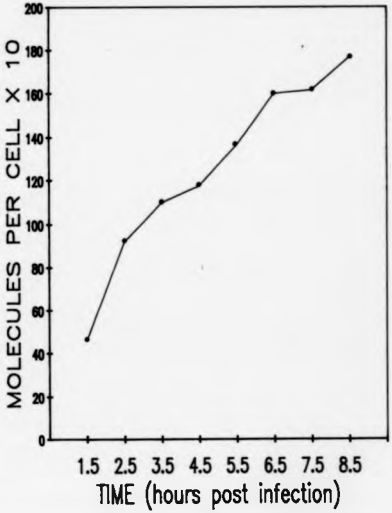


FIGURE 32. PART C: ACCUMULATION OF GENE 3 MINUS-SENSE (COMPLEMENTARY) RNA.

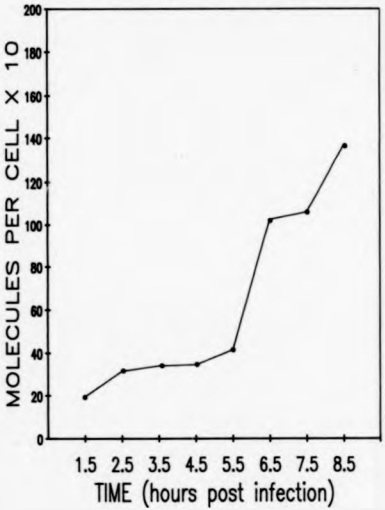


FIGURE 32. PART D: ACCUMULATION OF GENE 4 MINUS-SENSE (COMPLEMENTARY) RNA.

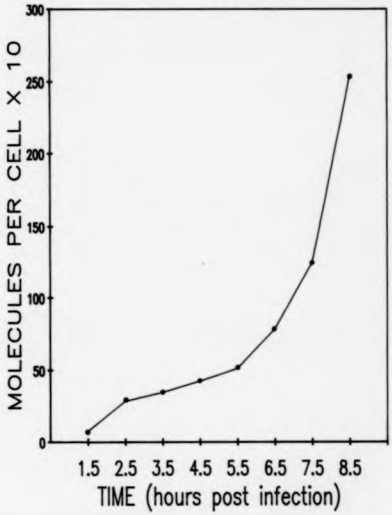


FIGURE 32. PART E: ACCUMULATION OF GENE 5 MINUS-SENSE (COMPLEMENTARY) RNA.

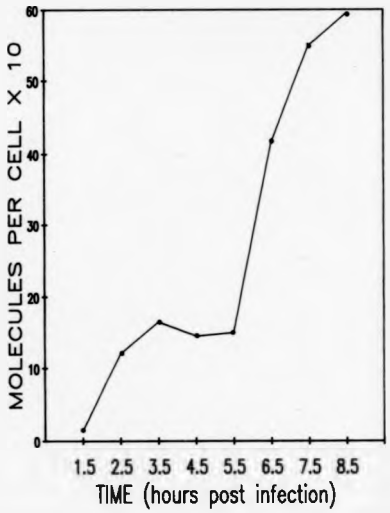


FIGURE 32. PART F: ACCUMULATION OF GENE 6 MINUS-SENSE (COMPLEMENTARY) RNA.

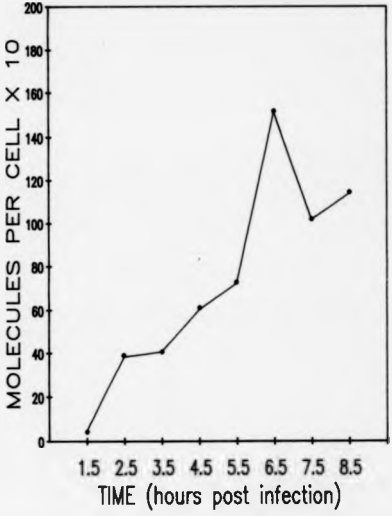


FIGURE 32. PART G: ACCUMULATION OF GENE 7 MINUS-SENSE (COMPLEMENTARY) RNA.

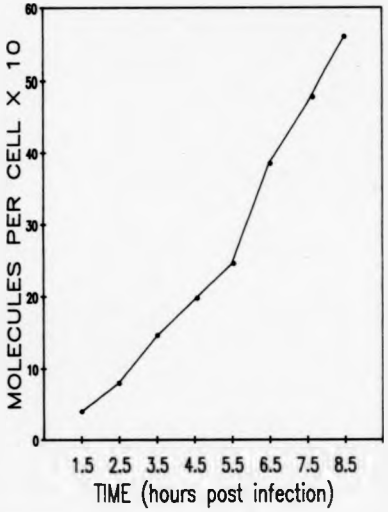


FIGURE 32. PART H: ACCUMULATION OF GENE 8 MINUS-SENSE (COMPLEMENTARY) RNA.

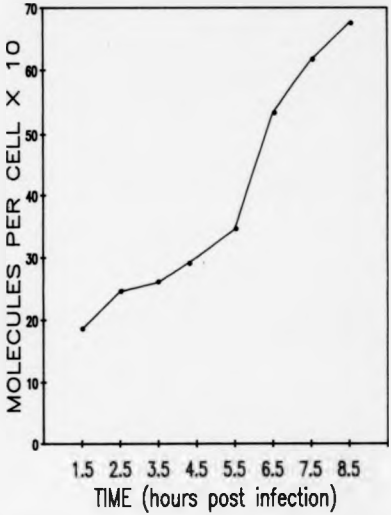


FIGURE 32. PART I: ACCUMULATION OF GENE 9 MINUS-SENSE (COMPLEMENTARY) RNA.

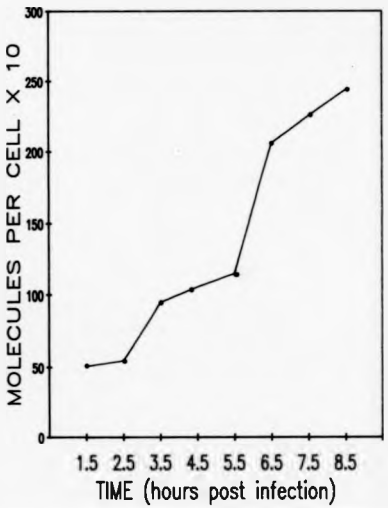


FIGURE 32. PART J: ACCUMULATION OF GENE 10 MINUS-SENSE (COMPLEMENTARY) RNA.

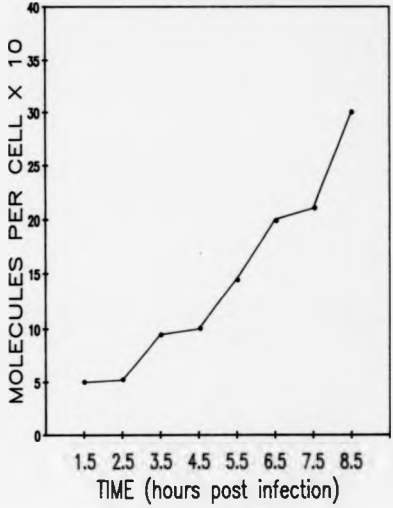


FIGURE 32. PART K: ACCUMULATION OF GENE 11 MINUS-SENSE (COMPLEMENTARY) RNA.

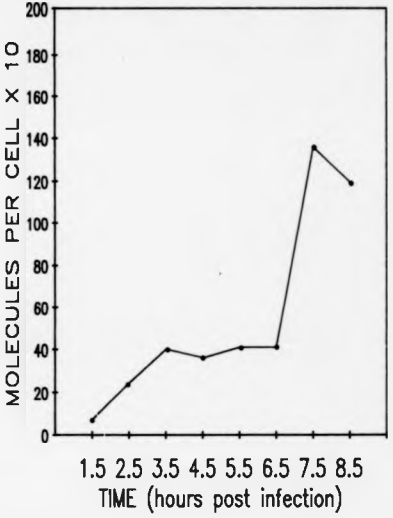
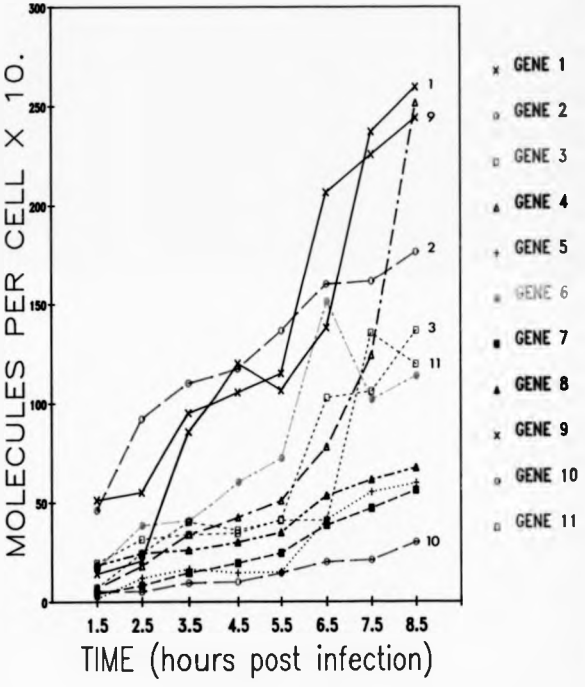


FIGURE 32 SUMMARY: ACCUMULATION OF MINUS SENSE RNA.



The data obtained for the accumulation of minus-sense RNA is presented in graphical form in Figure 32 parts A to K, and numerical data is appended in Table 16. It should be noted that the Y axes of Figure 32 are of a scale 100 X smaller than that of Figure 30 (for the accumulation of messenger RNA).

It is evident from the results presented in Figure 32 parts A-K that there were not equimolar amounts of the 11 minus-sense RNAs in infected cells. There was as much as an 8.5-fold excess of minus-sense RNA for Genes 1, 4 and 9 over that for Gene 10. Since the variability of the assay did not exceed 20%, it appears that this difference is a true one, and at face value would suggest that defective particles lacking one or more genome segments were being generated. The assumption that Rotavirus replication proceeds via a "non-conservative" mechanism precludes the possibility that this "excess" minus-strand RNA was free in the infected cells.

The genes could be divided into four groups according to the order in which the minus-sense RNA strands accumulated. Genes 2 and 9 minus-sense RNA began to accumulate very early in infection (1.5 hours PI) followed by Gene 1 at 3.5 hours PI. By 4.5, 6.5, and 7.5 hours P-I the accumulation of Genes 6, 3 and 4 and 11 respectively had begun, while accumulation of Genes 5, 7, 8 and 10 was slow by comparison, and reached the lowest final levels.

In view of the replication strategy established for Reovirus it was expected that minus-sense RNA accumulation would not begin until later in infection (production of progeny Rotavirions begins as early as 3.5 hours PI see section B). This appears to be true for 8 of the Genes, which either did not

accelerate their accumulation until after this time (eg. Genes 3, 4, 6 and 11) or increased slowly and steadily during infection (eg. Genes 5, 7, 8 and 10). However minus-sense RNA for genes 2 and 9 and to a lesser extent gene 1, accumulated rapidly early in infection. The reason for this observation is not clear.

B. GROWTH KINETICS DURING INFECTION

Figure 33 shows the production of infectious progeny Rotavirions expressed in terms of yield of plaque-forming units per cell. Under the conditions used (MOI=10 PFU/cell) the expected one-step growth curve was produced. After an initial eclipse phase (upto 3.5 hours) progeny virion production proceeded exponentially until 7.5 hours at which time a plateau was reached. The overall yield of infectious virus during this replication was 54 PFU/cell representing a five-fold amplification of input infectivity.

C. PARTICLE:PFU RATIO OF THE FINAL INFECTED CELL SAMPLE

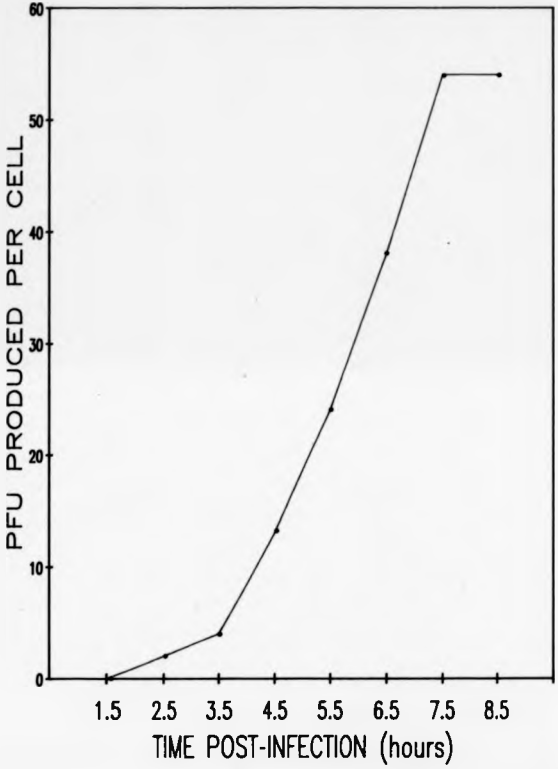
Measurement of the particle:PFU ratio was made at 8.5 hours post-infection. It was found that there were 4.7 recognisable virus particles per plaque-forming unit, which indicates that only 20% of the progeny particles were capable of causing infection.

DISCUSSION

FIGURE 33: ONE STEP GROWTH OF ROTAVIRUS: PRODUCTION OF INFECTIOUS PROGENY VIRIONS.

When infected cells were harvested at each time post-infection a sample was reserved for titration of infectivity. Virus yield in terms of PFU/cell was calculated and plotted versus time post-infection (hours). It is apparent that the cells were infected under single-step conditions as intended and that the yield of infectious virus was 54 PFU/cell.

FIGURE 33: ONE-STEP GROWTH OF ROTAVIRUS: PRODUCTION OF INFECTIOUS PROGENY VIRIONS.



It was found that the quantities of minus-sense RNA accumulated during the replication cycle were much lower (6-130 fold) than the quantities of plus-sense RNA (mRNA) found for the corresponding gene. This suggests that the majority (between 83 and 99%) of the plus-sense RNA molecules function only as template for protein synthesis, with the minority serving as a template for the addition of minus-sense RNA.

Reference to Figure 32 parts A-K shows that synthesis of the eleven minus-sense RNA strands was not simultaneous; it appeared that some genes such as genes 2 and 9 were replicated earlier than others. This apparent temporal division in accumulation of minus-sense RNA indicated that the genes may be linked during replication, since the complete absence of linkage would be reflected either in the simultaneous replication of the RNA molecules or in a synthetic order related to the size of the RNAs (smaller genes being accumulated more rapidly than the larger ones). Linkage of the mRNA molecules either prior to or during the replication process would allow sequential synthesis of the minus-sense RNA with the enzyme synthesising complementary RNA continuously, and genes would then be replicated in the order in which they were linked together. From studies on Reovirus sub-viral particles possessing ss → ds RNA polymerase activity, Zweerink (1974) suggested that RNA segments may be replicated in the order Small, Medium and then Large, implying some form of linkage between the mRNA segments within these particles. However, no such linkage of a covalent nature between mRNA molecules has been demonstrated either in

Reovirus (McCorquodale, personal communication) or Rotavirus (personal observation) and so if any form of linkage between RNA molecules does exist then it must be non-covalent.

Comparison of the quantities of minus-sense RNA accumulated with yield of infectious virus particles and the particle:PFU ratio shows that the percentage of the minus-sense RNA accounted for by virus particles varies between 9 and 84%. Gene 10 minus-sense RNA was produced in the smallest quantities at 8.5 hours pi. The yield of infectious virus particles at this time was 54 PFU/cell, which based on the particle:PFU ratio (4.7:1) measured represents a total of 254 molecules of gene 10 minus-sense RNA/cell. The actual quantity of gene 10 minus-sense RNA detected was 300 molecules per cell, which suggests that in the case of gene 10, 84% of the minus-sense RNA accumulated becomes part of a recognisable (by Electron Microscopy) Rotavirion. At the other extreme, only 9.8% of the gene 1 minus-sense RNA accumulated is present in complete virus particles. The accumulation of gene 10 minus-sense RNA corresponds well with that accountable for in terms of recognisable virus particles. Conversely there seems to be vast overproduction of gene 1, 4 and 9 minus-sense RNA. It is possible that at least some of the RNA was present in incomplete nascent virions which were either morphologically un-recognisable by E.M. or were so unstable in structure that they were destroyed during storage or preparation. To take these observations to their conclusion only approximately 2% of the Gene 1,4 and 9 minus-sense RNA accumulated became part of a virion capable of initiating an infection, suggesting that the replication process is extremely inefficient. Since these cells were infected at a very high

multiplicity of infection it is feasible that large numbers of defective particles may have been generated, containing complete copies of genes 1,4 and 9 but lacking one or more of the other genome segments, and being either morphologically distinct from complete virions or very unstable. Defective particles lacking all but the 5' and 3' termini of genome segments have already been observed in other virus systems such as Influenza. The existence of similar defective virions in Reovirus has been illustrated by Nonoyama and Graham (1970) who isolated Reovirus type 3 defective particles lacking the L1 gene (which encodes a polymerase protein). Defective Reovirions lacking other of the genome segments have also been identified (Ahmed and Graham 1977, Schuerch et al 1974). However to date defective virus particles have not been identified in the Rotavirus system. Hundley et al (1985) isolated Rotavirus particles lacking genome segment 5, but with rearranged RNA bands containing gene 5 sequences, by high multiplicity passage in tissue culture. Although these viruses failed to produce the normal gene 5 protein product on infection of tissue culture cells, all grew to a titer comparable to wild-type virus and were therefore not defective.

RESULTS: CHAPTER 7.

CHAPTER 7

QUALITATIVE REGULATION OF GENE EXPRESSION DURING VIRUS REPLICATION.

INTRODUCTION

In 1974 Nonoyama et al illustrated control of transcription during the replication of Reovirus type 3. By infecting L-cells in the presence of an inhibitor of protein synthesis (Cycloheximide) they were able to divide the Reovirus transcripts into two classes; "early" and "late". In the presence of cycloheximide only 4 of the 10 genes were transcribed, namely L1, M3, S3 and S4, which are now known to encode $\lambda 3$, μNS, σNS and $\sigma 3$ respectively (McCrae and Joklik 1978). Lau et al (1975) were able to show that the four transcripts were functional messenger RNA molecules. They infected L-cells with Reovirus type 3 and allowed the single-stranded transcripts listed above to accumulate for 17.5 hours in the presence of cycloheximide. The cycloheximide was then removed and the cells were exposed to radioactive amino acids to label any virus specific polypeptides which may be synthesised. Following immunoprecipitation of the viral polypeptides and fractionation by P.A.G.E. it was seen that within 30 minutes of cycloheximide removal four major polypeptides were synthesised. At this time the coding assignments of the 10 Reovirus genes had not been determined, but it is now known that these four polypeptides are the proteins encoded by genes L1, M3, S3 and S4 as described above (McCrae and Joklik 1978). By 3-4

hours after the removal of cycloheximide the pattern of protein synthesis had returned to normal, but the addition of cordycepin immediately after the removal of cycloheximide to inhibit the synthesis of new viral transcripts, resulted in the restricted pattern of protein synthesis being maintained. These authors concluded that the polypeptides produced immediately following cycloheximide removal were the translation products of RNA accumulated during the absence of protein synthesis, which must therefore have been functional mRNA. It was observed that in a non-permissive infection of L-cells with avian Reovirus only these same four genes were transcribed (Spandidos and Graham 1976) and that in coinfections of L-cells with Reovirus Type 3 and the Avian Reovirus, transcription of all 10 Avian genes occured although no replication of the genome was observed. It appeared, from these results that one or more of the early gene products was required for transcription of the rest of the genome. It was suggested that in infected cells a cellular protein was responsible for repressing viral transcription and that one of the early viral gene products was capable of inactivating it, thereby functioning as a de-repressor. The early gene products as described previously (Introduction Section 2,G) are λ3 and μns, which have both been implicated in genome replication (Dryna and Fields 1982, Gomatos et al 1981), σ3 which has been shown to be involved in inhibition of host-cell protein synthesis (Sharpe and Fields 1981,1982), and σ NS which is known to bind specifically to single-stranded RNA. It is possible that these four early mRNAs are transcribed early in infection and are not subject to any host-cell mediated transcriptional repression. Further it is postulated that one or more of the gene products acts as a "de-repressor" allowing the

transcription of the complete genome. However since no such host-cell repressor protein has been identified, the possibilities outlined above have yet to be confirmed.

We wished to determine whether, in a similar fashion to Reovirus, there was any qualitative regulation of gene expression during Rotavirus replication. The method used was essentially the same as that described by Lau et al (1975) with adjustments for the length of the Rotavirus replication cycle. Briefly, cycloheximide was added to cell monolayers (1 million cells) at a concentration of 20 μg/ml two hours prior to infection with Rotavirus under single-step growth conditions. Infection was allowed to proceed at 37° c in the presence of the inhibitor until 6.5 hours PI at which time the cycloheximide was removed, and cells were pulse-labelled for 15 minutes with ^{35}-S methionine at 15 minutes and 3 hours after removal. Samples were balanced with respect to precipitable ^{35}-S and labelled polypeptides were examined by P.A.G.E. Initially protein profiles were examined with a view to identification of any protein products produced only 15 minutes following cycloheximide removal. This was followed by study of the accumulation of the corresponding messenger RNAs. Cycloheximide treated cells for RNA analysis were prepared separately from those used for the protein analysis, and were prelabelled with ^{3}-H Uridine exactly as described for the cells used in RNA accumulation analysis (see Methods Section 2 and Chapter 5). Following extraction of the RNA from these cells, the incorporated ^{3}-H was measured by TCA precipitation. This allowed the direct comparison of mRNA levels detected in cycloheximide treated cells, with those detected in the untreated

cells at the same time PI (see Chapter 5), as the extracted samples could be adjusted to contain the same number of cell equivalents of RNA per unit volume.

RESULTS

A. EFFECTIVENESS OF CYCLOHEXIMIDE

To confirm that the cycloheximide was effective at the concentration used, the incorporation of ^{35}-S Methionine into each sample was measured by TCA precipitation. Table 13 shows the incorporation of radioactivity into the five samples. It was evident from these results that 20ug/ml cycloheximide effectively inhibited protein synthesis in uninfected BSC-1 cells; treated cells incorporated only 9.8% of the ^{35}-S methionine incorporated by the untreated cells. Infection with Rotavirus decreased overall protein synthesis in untreated cells by 55% which was due to the effective host-cell inhibition operating at this time in infection. Because of the large reduction in protein synthesis caused by the infection, the infected cell results were used in calculation of recovery of protein synthesis following cycloheximide removal (lower portion of Table 13). Cells allowed to recover from cycloheximide treatment for 15 minutes before pulse-labelling showed only a 63% recovery in protein synthesis, however when cells were allowed to recover for three hours protein synthesis returned to normal.

TABLE 13: INHIBITION OF PROTEIN SYNTHESIS BY CYCLOHEXIMIDE.

Cycloheximide-treated infected cells were prepared as described in the text. 100ul aliquots were applied to Whatman 3MM filter paper discs and treated with hot TCA, to precipitate proteins (see Methods section 9). The TCA precipitable radioactivity in each sample was then used to calculate the reduction in protein synthesis caused by the cycloheximide. The upper portion of Table 13 illustrates the effect of cycloheximide, and of Rotavirus infection on protein synthesis. Cycloheximide at a concentration of 20ug/ml reduced protein synthesis in uninfected cells to 9.8% showing that it was 90% effective. Rotavirus infection itself caused the level of protein synthesis in untreated cells to fall to 45%, which was due to the effective host-cell inhibition operating at this time PI. Because of the virus induced inhibition it was appropriate to use the infected cell control as the standard when calculating the recovery of protein synthesis following the removal of the cycloheximide. The lower portion of Table 13 shows that 15 minutes after removal of cycloheximide protein synthesis in infected cells has recovered to 63% of that seen in untreated infected cells, and by 3 hours the recovery is complete.

SAMPLE	CPM 35-S METHIONINE INCORPORATED IN 15 MINUTE PULSE	AS A PERCENTAGE OF APPROPRIATE CONTROL
UNINFECTED CONTROL	16,596	100%
INFECTED CONTROL	7,470	45%
UNINFECTED + CYCLOHEXIMIDE	1,640	9.8%
INFECTED CONTROL	7,470	100%
INFECTED CELLS PULSE-LABELLED 15 MINUTES AFTER CYCLOHEXIMIDE REMOVAL	4,693	63%
INFECTED CELLS PULSE-LABELLED 3 HOURS AFTER CYCLOHEXIMIDE REMOVAL	7,929	>100%

B. SELECTIVE TRANSCRIPTION IN THE ABSENCE OF PROTEIN SYNTHESIS

P.A.G.E. analysis of the pulse-labelled samples is shown in Figure 34. As early as 15 minutes after the removal of cycloheximide 4 of the Rotavirus encoded proteins could be detected; VP5, VP6, VP8 and VP9. VP9 and VP5 appeared to be present at near normal levels (an observation made solely on the intensities of the bands) while VP6 and VP8 did not appear to have recovered to their normal level of expression during this time. The production of these 4 proteins so soon after cycloheximide removal implies that transcription of the genes encoding these 4 proteins (Genes 5, 6, 7 and 9) was independent of protein synthesis. The quantities of viral RNA present in cycloheximide treated cells should therefore reflect the observed pattern of protein synthesis. RNA was extracted from the cells prepared for this purpose (as described in the Introduction to this chapter) and accumulation of mRNA for Genes 5, 6, 7 and 9 analysed by the Solution Hybridisation assay (see Chapter 2). It was then possible to compare the transcription of Genes 5, 6, 7 and 9 with those of Genes 3 an 10 (since VP3 and VP10 were absent from the protein profile at 15 minutes after cycloheximide removal they were used as controls) in the cycloheximide treated cells and the untreated cells.

Table 14 shows the results of this comparison; molecules of RNA accumulated in the untreated cells is compared to that in the treated cells, and the fold reduction calculated for each set of data. The production of protein following the removal of cycloheximide was reflected in the accumulation of the

FIGURE 34: SELECTIVE TRANSCRIPTION IN THE ABSENSE OF HOST-CELL PROTEIN SYNTHESIS.

Cycloheximide treated infected cells were pulse labelled for 15 minutes at 6.5 hours post-infection with 35-S methionine as described in the text, and then fractionated on 5-11% polyacrylamide gels using the untreated Rotavirus protein profile as marker. It was possible to identify 4 proteins synthesised only 15 minutes following the removal of cycloheximide, indicating that the transcription of the corresponding mRNAs was independent of protein synthesis.

LANE A: Cycloheximide treated uninfected cells.

LANE B: Untreated uninfected cells.

LANE C: Untreated infected cells at 6.5 hours PI.

LANE D: Cycloheximide treated infected cells labelled 15 minutes after removal.

LANE E: Cycloheximide treated infected cells labelled 3 hours after removal.

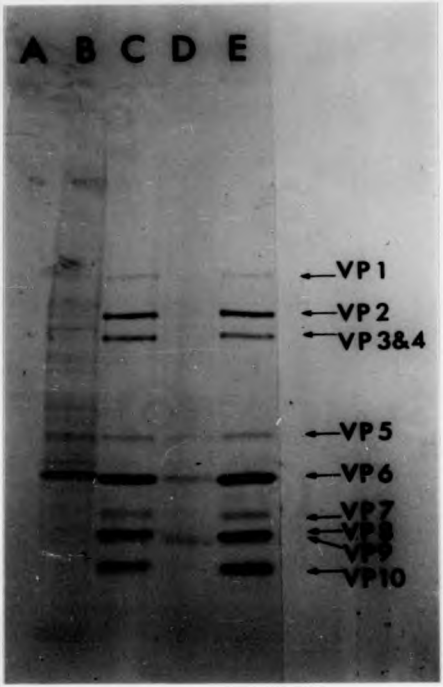

A B C D E
VP1
VP2
VP3&4
VP5
VP6
VP7
VP8
VP9
VP10

TABLE 14: mRNA ACCUMULATION IN THE PRESENCE OF CYCLOHEXIMIDE.

Accumulation of six of the mRNA species were compared in the presence and absence of protein synthesis. The four genes encoding the polypeptides visible in Figure 34 were compared with two which were not visible (genes 3 and 10). It is evident that in the presence of cycloheximide accumulation of all six mRNAs is reduced, but that the reduction is greater in genes 3 and 10 than for gene 5, 6, 7 and 9 indicating that transcription of these genes is independant of protein synthesis.

	MOLECULES OF	RNA ACCUMULATED	PER CELL
GENE	UNTREATED CELLS	TREATED CELLS	FOLD REDUCTION
SP5	20,470	15,300	1.3
SP6	15,795	2,870	5.5
SP7	63,900	12,500	5.1
SP9	21,180	3,290	6.4
SP3	28,400	835	34.0
SP10	16,960	1,300	13.0

mRNA prior to the release. All of the genes examined showed a reduction in mRNA accumulation in the presence of cycloheximide, but it was apparent that the genes expressed under these conditions showed a much smaller reduction than those which were not detected. For example the accumulation of mRNA for Genes 5, 6, 7 and 9 whose protein products are produced in the 15 minutes after cycloheximide removal are reduced by only 1.5 to 6.5 fold (accumulation is between 15 and 19% (Genes 6, 7 and 9) and 74% (Gene 5) of the accumulation in untreated cells) while accumulation of mRNA for Genes 3 and 10, whose products are not visible by P.A.G.E. were reduced much more, being accumulated to only 3 and 7.5% respectively of the quantities detected in untreated cells.

DISCUSSION

The results obtained in Chapter 7 indicated that transcription of 4 of the 11 Rotavirus genes was independent of protein synthesis, while production of the remaining 7 mRNA species was dependent upon it. These observations suggest that these "early" gene products (VP5, VP6 VP8 and VP9) may play a regulatory role in early infection as was suggested for the four Reovirus gene products synthesised in the absence of protein synthesis (See introduction to this Chapter). Although the data presented here cannot give any indication of the nature of such roles for the 4 proteins concerned, it is possible to speculate as to their possible functions based on their physical and biochemical properties.

VP5 and VP6 are both structural proteins, VP6 is located on the inner shell of the virion, while the exact location of VP5 remains to be confirmed. Sandino et al (1986) have associated VP6 with the ability of viral cores to carry out transcription (see Introduction Section 1iii), it is possible that the newly synthesised SP6 is able to interact with the proteins present in the Rotavirus Sub-Viral particle and in some way allow transcription of the entire genome. VP9 is a non-structural protein about which little is known. VP8 is thought by some groups to be non-structural in nature, but in the UKtc system is probably a structural protein (McCrae and Faulkner-Valle 1981) and is produced in relatively large quantities during replication (Chapter 5). The RNA binding properties of this protein (McCrae and Baybutt personal communication) support the proposal that VP8 may have a role in the regulation of transcription: for example, VP8 could regulate transcription of the RNA genome by binding to and blocking the transcriptase binding sites of each segment, this suggestion depends on the assumption that VP8 is small enough to gain access to the RNA by passage into the viral cores. If a cellular repressor of viral transcription is shown to be involved in Rotavirus replication (as was suggested for Reovirus), it is possible that any one of the four proteins discussed above could act as a de-repressor.

Although these mRNA species are transcribed independently of protein synthesis, they do not appear to accumulate early during the course of a normal one-step infection, and so their regulation of transcription (if this is what we

are observing) is more subtle that the simple "early-late" switches seen in other viruses such as polyoma virus.

GENERAL DISCUSSION.

GENERAL DISCUSSION.

The aim of the work presented in this thesis was to develop a method to molecularly dissect the synthesis of Rotavirus RNA. This has been achieved by the use of single-stranded RNA probes in a newly developed Solution Hybridisation assay, which has allowed the accurate quantitation of both strands of all 11 Rotavirus genes during an 8.5 hour simultaneous infection in tissue culture. It has already been pointed out (Chapter 5) that the RNA levels measured by this assay are cumulative in nature; they represent the net accumulation of RNA, which results from the balance between synthesis and decay for each gene. A major shortcoming of the assay was therefore its failure to consider potential differential decay rates of the RNA species. As an example, Gene 7 mRNA was accumulated in more than twice the quantity of Gene 8 mRNA, by 7.5 hours PI (see Chapter 5 and Table 15) and the assay in its present form does not indicate whether this difference was due to a higher rate of synthesis of Gene 7 mRNA, or a higher rate of decay of Gene 8 mRNA. Several attempts have been made to determine the relative decay rates of Genes 7 and 8 mRNA , these were chosen because of the large difference in their levels of accumulation.

Cells were infected at an MOI of 10 PFU per cell, and at 2 and 6 hours post infection, pulse-labelled with ^{3}-H Uridine for 30 minutes, to label newly synthesised viral RNA. The label was then replaced with medium and incubation continued for a further 2 hours, cells being harvested at 20 minute intervals throughout this time. Thus at both an "early" time (2 hours) and

a "late" time (6 hours) PI there were 6 chase samples of the RNA labelled during the pulse. The labelled RNA was then extracted from these cells, and hybridised to an excess of "cold" minus-sense RNA (the pg amount of RNA needed to achieve an excess was calculated based on the results presented in Chapter 5) to "capture" the ^{3}-H labelled RNA. It should have been possible to follow viral RNA labelled in the pulse for the 2 hour period and determine whether decay of RNA was occurring, and if so was the rate of decay the same for both genes examined? However, no conclusions were reached for two reasons; 1) It was not possible to introduce sufficient ^{3}-H into the viral RNA during a 30 minute pulse to make measuring its decay feasible. ^{3}-H uridine was used at the highest specific activity available (40-60 Ci/mMol). 2) In the infected cell samples prepared for RNA analysis (see Chapters 5 and 6) cells had been pre-labelled with ^{3}-H uridine so that the extracted samples could be balanced with respect to cellular RNA content. In this case, ^{3}-H uridine could not be used for pre-labelling as the cells were being pulsed-labelled with this isotope. RNA samples were balanced by OD-260 and also by pre-labelling with a very small amount of ^{32}-P (carrier free). However neither method proved satisfactory, and so to date no conclusion has been reached regarding the decay of viral RNA during the infection studied.

It was noted in Chapter 6 that there was a large molar excess of some of the minus-sense RNA species (eg. Genes 1,4 and 9) over others (eg.Gene 10). This suggested that during the course of the infection, which was performed using a high MOI (10 PFU/cell) defective Rotavirus particles were produced. Virus particles lacking their full complement of normal

genome segments have already been isolated from high MOI Rotavirus infections but were found to be non-defective (Hundley et al 1985). As was illustrated from the yield of infectious virus in Chapter 6, any virus particles with an incomplete genome generated in the present infection must be non-infectious. Based on the assumption that such particles would deviate from the normal particle density of ρ=1.36 we intended to fractionate infected cells on a caesium chloride gradient, and analyse the Gene 4 and 10 minus-sense content across this gradient. It was predicted that infectious virus would contain a 1:1 molar quantity of all minus-sense RNA species, while any virions lacking genome segments and equilibrating elsewhere on the gradient, would deviate from such an equimolar relationship. An 8.5 hour infected cell sample was prepared and treated with detergents, sonication and homogenisation to break up the cells. The homogenate was then loaded onto a pre-formed caesium chloride gradient (ρ= 1.2 to 1.4) and centrifuged to equilibrium. 0.5 ml fractions were collected by side puncture at the bottom of the tube, and dialysed against dilute buffer to remove the CsCl. Aliquots were then titrated on monolayers of BSC-1 cells and it was possible to plot a graph of infectivity versus fraction position. When the results of the titration were analysed, it became clear that this approach to the problem was not ideal. Firstly, approximately 60% of the infectious virus in the samples equilibrated to ρ=1.36, while the remaining 40% remained associated with cellular material (such as membranes) towards the top of the gradient. Several different regimens of detergent treatment were used in an attempt to dissociate the infectious virus from the cell components, but none were completely

successful. Secondly, the level of infectivity on the gradient was insufficient to permit detection of the RNA even by Solution Hybridisation. For example, at the infectivity peak on the gradient there were only 1 x 10^6 PFU/ml fraction. A minimum of 10 pg of RNA is needed for detection in this assay which would be present in 3.15 X 10^7 PFU (for gene 4) and 8.31 X 10^7 PFU (for gene 10), so in order to use the solution hybridisation assay in this way at least 100 times more virus infectivity was needed, as the hybridisation would have to be repeated several times. These considerations therefore make this approach impractical.

A second possible explanation for the accumulation of a large excess of some minus-sense RNAs relative to others, is that many nascent virus particles fail to reach maturity. For example, progeny virions may reach the stage of morphogenesis where the mRNA molecules are assembled with viral protein, and begin to replicate their genome by the synthesis of minus-sense RNA, but fail to complete the replication. Such particles would probably either be morphologically unrecognisable as Rotavirions, or be so unstable that they would become degraded during storage or preparation of the E.M. grids. In support of this, Chapter 6 and Table 16 (Appendix) show that of the four minus-sense RNAs accumulated in a large excess over gene 10 (genes 1, 2, 4 and 9) three are also accumulated early (by 3.5 hours PI) in infection (genes 1, 2 and 9). This hypothesis would therefore predict an order for gene replication as was discussed in Chapter 6 (discussion).

COMPARISON OF THE SOLUTION HYBRIDISATION ASSAY WITH A DOT-BLOT BASED ASSAY.

When we began developing this assay, the dot-blot methodology had already been in use in the laboratory for some time, and had been successfully used by Piwnica-Worms and Keene (1985) to study the replication of Vesicular Stomatitis Virus. However, as outlined in Chapter 3 we felt that a Solution Hybridisation would give us a more sensitive, more quantifiable and rapid means of studying viral replication. Comparison of the new Solution Hybridisation method with a Dot-Blot based assay (see Chapter 4 and Piwnica-Worms and Keene 1985) indicated that solution hybridisation was at least 6 times more sensitive than the Dot-blot assay (derived by comparison of the amount of RNA required for the detection of 64,000 molecules of Gene 7 mRNA per cell at 6.5 hours post infection) using the single-stranded RNA probes. In the Dot-Blot assay described in Chapter 4 (page 161) a clear positive signal was obtained with 1ng of complementary RNA (Figure 28, Row 1, column F). By using the Solution Hybridisation assay as little as 10pg of viral RNA could be accurately measured. The Solution Hybridisation method therefore gave a 100 fold increase in sensitivity over Dot-blotting when the amount of RNA required for the assay was considered. This point becomes of particular importance when a study such as the one described in this thesis is undertaken, where all of the hybridisation assays had to be carried out on the same RNA stocks to ensure direct comparability of all the results. By Dot-blot analysis, Gene 7 minus-sense RNA could not be detected in as many as 3 million cell equivalents (Figure 28, Row 6) whereas by solution hybridisation only 1 million cells were required. The small area of the Nylon membrane which is available to bind RNA may play a part in the reduced sensitivity

of the Dot-blotting method. For the Biorad "Biodot" apparatus which was used for the preparation of the blots shown in Figures 28 and 29, this was 19.5 square mm. It is possible that in Dot-Blotting very uncommon viral RNA sequences would be undetected, since the large amounts of cellular RNA also present in the large sample needed for its detection may "mask" the viral RNA, or prevent it from binding to the dot (by competition) so that it was present at a level below the minumum required for detection. By contrast in solution it may be that more of the RNA was available for hybridisation with the probe RNA, due to the absence of immobilisation. However this last argument also points out one of the shortcomings of this assay, and one alluded to by Piwnica-Worms and Keene (1985) which is that in Dot-Blotting the RNA is immobilised, thus preventing any reannealing of complementary RNA such as dsRNA genomes, to the exclusion of the probe. With the Solution Hybridisation method the possibility that this may be occurring in some cases must be considered. When the probe was added to the hybridisation it was in a molar excess over the complementary RNA but not necessarily over RNA of the same sense. In the case of messenger RNA detection assays this was almost certainly not a problem since the minus sense RNA (as illustrated in Chapters 5 and 6) was present in very much smaller quantities than the messenger RNA, and would therefore be present as only a small proportion of the total minus-sense sequences (probe RNA plus viral sequence). Conversely, when detecting the minus-sense strand of a gene using a plus-sense probe, the viral messenger RNA was more likely to cause interference with the hybridisation. For example for gene 11, 500,000 CPM (or 330 pg at the specific activity used) of probe RNA was equivalent to 1

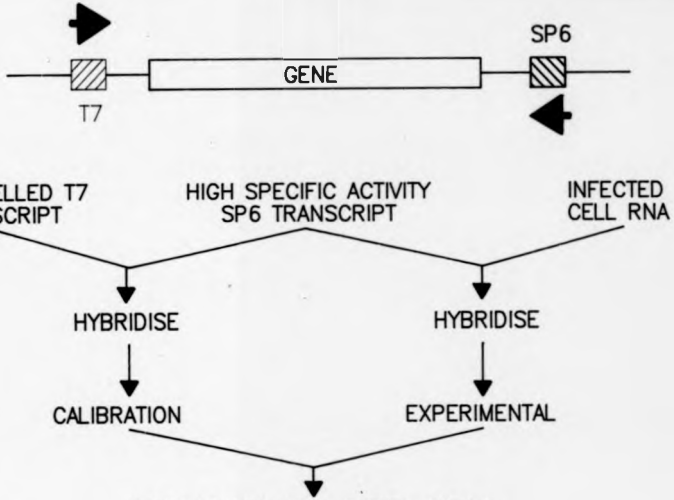
x 10^9 molecules of Gene 11 RNA. If this was being added to 1 x 10^6 cells to detect minus sense RNA at a time when there are also 215 molecules of gene 11 Messenger RNA present per cell (1.5 hours PI total 2.15 x 10^8 molecules) then the total plus-sense RNA present in the hybridisation consists of 80% probe and 20% Gene 11 mRNA. This 20% of cold complementary RNA could potentially reduce the amount of minus-sense RNA detected, and will vary with time post-infection depending on how much mRNA there is per cell. If the events described above do significantly affect the levels of minus sense RNA detected then it would be expected that those genes having a very high level of messenger RNA would show lower levels of minus-sense RNA since there would be more competing RNA present in the hybridisation. However it is the general rule (see Table 15; Appendix) that genes with a high accumulation of mRNA also have high levels of minus-sense RNA and so it is supposed that this effect is not significant although the possibility that the minus-sense RNA is being under-detected should not be ignored. The assay could therefore be improved by the selective removal of the messenger RNA before hybridisation to detect minus-sense RNA.

FINAL CONCLUSIONS

The Solution Hybridisation method developed is summarised in Figure 35. By making single stranded RNA copies of both strands of cDNA clones of the 11 Rotavirus genes, it has been possible to accurately and sensitively detect the accumulation of Rotavirus RNA throughout the 8.5 hours infection cycle studied. The Solution Hybridisation assay has a limit of detection which can be as little as 5 molecules of RNA per cell

FIGURE 35: SUMMARY OF THE SOLUTION HYBRIDISATION ASSAY FOR THE ACCUMULATION OF SPECIFIC STRANDS OF INDIVIDUAL RNA SPECIES DURING A ROTAVIRUS INFECTION.

Following the insertion of cloned c-DNA sequences into the Gemini vectors, high specific activity RNA copies of either strand were generated using T7 or SP6 polymerase. This "probe" RNA was then hybridised to both known amounts of complementary transcript (calibration) and RNA extracted from infected cells (experimental). Following removal of unhybridised RNA by digestion with RNases the amount of probe-specific RNA in a sample could be calculated from its RNase resistant radioactivity. Results are finally expressed in terms of molecules of RNA per cell.


SP6
GENE
T7
UNLABELLED T7 TRANSCRIPT
HIGH SPECIFIC ACTIVITY SP6 TRANSCRIPT
INFECTED CELL RNA
HYBRIDISE
HYBRIDISE
CALIBRATION
EXPERIMENTAL
MOLECULES PER CELL

depending on the size of the probe relative to the gene from which it was derived and the number of cells put into the hybridisation. The results presented indicate that Rotavirus replication is likely to proceed in a similar fashion to Reovirus, that is non-conservatively, although this has not been shown conclusively (in addition, the type of analysis described here has not yet been carried out on Reovirus). Several factors point towards this probability: i) Messenger RNA accumulated to a high level early in infection (upto 3.5 hours PI) while minus sense RNA accumulates slowly and steadily throughout, suggesting that minus-sense RNA did not act as a replicative intermediate. ii) Although large differences were seen between the amounts of minus-sense RNA of the different genes accumulated, the results obtained for gene 10 when combined with the particle:PFU ratio indicated that most of the minus-sense RNA synthesised became part of a recognizable (by EM) Rotavirion. This suggested that minus-sense RNA is probably not found free in the cell cytoplasm. iii) Following the appearance of progeny virions at 2.5-3.5 hours post infection there was a rise in the rate of mRNA accumulation of all genes (except Gene 10). This suggested that progeny virions served as a template for production of more mRNA. Before 2.5 hours post infection mRNA was detected at a level within 2-5 fold of the minus-sense RNA (note that the cells were washed following adsorption and so this does not represent viral inoculum). Thereafter the magnitude of the difference between plus and minus sense strands of all the Genes continued to increase. Using protein synthesis studies in conjunction with the RNA data it has been possible to illustrate the operation of both quantitative and qualitative transcriptional and translational control over gene

expression during Rotavirus replication.

In 1974 transcription of the reovirus genome was studied by Nonoyama et al whose work has formed the basis of the present study of Rotavirus. They labelled mRNA transcribed in vivo with ^{3}H-uridine, extracted it from the infected cells and hybridised to an excess of genomic dsRNA labelled with 2-^{14}C uridine. The dsRNA hybrids were then fractionated by PAGE. By measuring the radioactivity contained in the transcript-genome hybrids these authors were able to calculate the relative frequencies of transcription of the 10 species of mRNA at different times post-infection (cycloheximide was used to "freeze" the pattern of transcription at the required time PI). It became apparent from this series of experiments that at late times post-infection the reovirus genome segments were transcribed at equal frequencies, but at earlier times only subsets of the viral genes were transcriptionally active. Four of the genes (see Chapter 7) were transcribed early in infection (prior to 4 hours) and the remaining six genes appeared to become transcriptionally active in a sequential fashion (Nonoyama et al 1974).

The method described in this manuscript offers several advantages over the system used by Nonoyama et al (1974) many of which are a result of the great advances made in molecular biology during the last decade. Firstly, the present method does not require that the infected cells are radioactively labelled to a high specific activity during infection (as with the system of Nonoyama et al 1974) which greatly facilitated the preparation of infected cell RNA and enabled large quantities of RNA required for comparative

analysis of the different genes to be stored for long periods of time without the risk of radiolytic degradation. In addition Nonoyama et al (1974) used radioactively labelled genomic RNA used to "capture" the labelled viral transcripts thereby requiring the preparation of large amounts of purified virus from which to obtain the viral genome. It was much quicker and more convenient to synthesise large amounts of un-labelled virus specific transcript RNA using T7 or SP6 polymerase in vitro, for this purpose. Secondly because of the experimental design employed by Nonoyama et al (1974) each set of data obtained which often represented transcription at different times PI, was obtained from a separate set of infected cell samples. In the present study all data was directly comparable since the samples for each time-point were obtained from the same infection event. Thirdly, again due to the experimental design, Nonoyama et al (1974) did not use their method to measure the replication of the ds-RNA genomes of reovirus. It is doubtful, given the low levels of minus-sense RNA accumulated during the replication of Rotavirus (Chapter 6, this manuscript) that genome replication could have been accurately measured by a method involving in vivo labelling. Finally although the method of Nonoyama et al (1974) gives relative transcriptional frequencies for the 10 reovirus genes it is unable to estimate the absolute numbers of molecules of RNA synthesised per cell, since their calculations are based on the ratios of ^{3}H-u (in mRNA) to ^{14}C-u (from the genomic RNA) in the hybrids formed, and are not related to the amount of RNA (in terms of cell equivalents) put into the hybridisation assay. However,

the method of Nonoyama et al (1974) does allow actual RNA synthesis to be measured over a short (1 hour) period of time, whereas the method described in this manuscript measured the net accumulation of RNA up to the time of sampling.

All the results presented in this manuscript were obtained from one experimental infection, which was essential to allow comparison of data for each of the 11 genes. Preliminary experiments on a separate set of infected cells samples have shown that the ratios of transcription and replication of the different genome segments are similar under this defined set of infection conditions (37°C and an MOI of 10 PFU/cell).

APPENDIX.

TABLES 15 AND 16 PARTS A-K: ACCUMULATION OF PLUS AND MINUS SENSE RNA FOR THE 11 ROTAVIRUS GENES.

The accumulation of minus-sense RNA was measured for each of the 11 Rotavirus genes at hourly intervals (as indicated) throughout an 8.5 hour simultaneous infection as previously described. The results obtained for genes 1 to 11 are labelled A through K respectively. For each gene the hybridization assay was repeated until three sets of data having all infected cell samples on the linear part of the calibration curve had been obtained (see Chapters 5 and 6).

TABLE 15 PARTS A - K.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<120	<120	<120	<120
1.5 HOURS	740	880	770	780
2.5 HOURS	1710	2080	1520	1880
3.5 HOURS	14760	16800	13880	15070
4.5 HOURS	18300	17580	20400	18080
5.5 HOURS	28700	20470	23680	23300
6.5 HOURS	28800	24370	31800	28880
7.5 HOURS	32000	28780	33700	31820
8.5 HOURS	34880	33480	37850	36300

TABLE 15Ai ACCUMULATION OF GENE 1 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<120	<120	<120	<120
1.5 HOURS	790	1040	880	888
2.5 HOURS	2235	2885	2480	2530
3.5 HOURS	14310	16480	18780	15530
4.5 HOURS	34828	31740	33800	33330
5.5 HOURS	33780	38778	38780	38438
6.5 HOURS	52186	48478	51480	50700
7.5 HOURS	58280	63840	58880	60738
8.5 HOURS	84840	83480	84100	84078

TABLE 15Bi ACCUMULATION OF GENE 2 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<80	<80	<80	<80
1.5 HOURS	280	280	320	288
2.5 HOURS	1680	1280	880	1280
3.5 HOURS	4840	4750	6320	5300
4.5 HOURS	7880	8080	8280	8778
5.5 HOURS	18780	17840	15820	16408
6.5 HOURS	27080	28648	28480	28400
7.5 HOURS	50078	48400	48130	48200
8.5 HOURS	48480	42888	48118	44808

TABLE 18b: ACCUMULATION OF GENE 3 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<80	<80	<80	<80
1.5 HOURS	530	480	510	520
2.5 HOURS	580	530	580	550
3.5 HOURS	7240	7680	7430	7480
4.5 HOURS	8730	8580	8760	8680
5.5 HOURS	11310	10288	12280	11300
6.5 HOURS	11700	12800	12580	12280
7.5 HOURS	22088	20088	23870	22010
8.5 HOURS	13880	15400	15120	14700

TABLE 19b: ACCUMULATION OF GENE 4 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<45	<45	<45	<45
1.5 HOURS	440	370	380	400
2.5 HOURS	880	880	840	840
3.5 HOURS	8000	10850	7388	8780
4.5 HOURS	11780	9880	10480	10688
5.5 HOURS	11780	13000	12410	12388
6.5 HOURS	21720	19840	20180	20470
7.5 HOURS	30400	24700	27430	27810
8.5 HOURS	28000	33440	31400	30280

TABLE 18D) ACCUMULATION OF GENE 8 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	3	4	MOLECULES PER CELL 5	6	MEAN
UNINFECTED	<50	<50	<50	<50	<50	<50	<50
1.5 HOURS	250	280	235	240	270	285	255
2.5 HOURS	500	420	525	455	630	485	500
3.5 HOURS	10990	9480	10485	9210	9810	10115	9980
4.5 HOURS	14340	12480	13920	14110	13310	10880	13130
5.5 HOURS	15295	15110	14790	15140	13880	14340	14710
6.5 HOURS	13380	16250	16420	15870	16440	15300	15795
7.5 HOURS	21700	22480	23400	21500	24480	24380	22975
8.5 HOURS	21100	21780	22500	20100	22980	21710	21690

TABLE 18E) ACCUMULATION OF GENE 8 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<200	<200	<200	<200
1.5 HOURS	630	756	888	828
2.5 HOURS	930	1180	1065	1050
3.5 HOURS	11740	14840	13635	13405
4.5 HOURS	37810	40040	46680	41200
5.5 HOURS	68750	68120	78330	71066
6.5 HOURS	57600	68380	64730	63600
7.5 HOURS	80460	73118	78840	80800
8.5 HOURS	66420	78348	78840	73636

TABLE 19b: ACCUMULATION OF GENE 7 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<90	<90	<90	<90
1.5 HOURS	340	370	410	378
2.5 HOURS	1000	1480	1200	1216
3.5 HOURS	7100	8000	7668	7668
4.5 HOURS	18600	18470	18300	18190
5.5 HOURS	20668	19870	21445	20640
6.5 HOURS	22760	21780	20180	21660
7.5 HOURS	33400	32760	31400	32518
8.5 HOURS	30660	34660	31370	32168

TABLE 19c: ACCUMULATION OF GENE 8 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<65	<65	<65	<65
1.5 HOURS	200	225	238	220
2.5 HOURS	700	830	745	760
3.5 HOURS	2475	2745	2885	2730
4.5 HOURS	3850	3885	3850	3885
5.5 HOURS	4520	4485	3825	4315
6.5 HOURS	20760	21420	21360	21180
7.5 HOURS	28445	28730	28430	28200
8.5 HOURS	36750	34810	36240	36935

TABLE 18: ACCUMULATION OF GENE 8 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
[illegible]	<10	<10	<10	<10
1.5 HOURS	85	80	100	90
2.5 HOURS	200	230	230	215
3.5 HOURS	3100	3080	3230	3080
4.5 HOURS	6130	6540	6680	6200
5.5 HOURS	7100	7800	7580	7488
6.5 HOURS	16620	16680	17100	16860
7.5 HOURS	22480	23000	22180	22848
8.5 HOURS	17000	20600	18810	18840

TABLE 19: ACCUMULATION OF GENE 10 PLUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / MOLECULES PER CELL			
	1	2	3	MEAN
UNINFECTED	<170	<170	<170	<170
1.5 HOURS	240	190	220	215
2.5 HOURS	545	540	580	580
3.5 HOURS	7480	8455	8250	8068
4.5 HOURS	14880	15880	15410	15560
5.5 HOURS	18040	17575	17100	17870
6.5 HOURS	21500	23340	22380	23060
7.5 HOURS	34725	38040	36480	36405
8.5 HOURS	32680	35830	34180	34220

TABLE 18G ACCUMULATION OF 60S 11 PLUS-SENSE RNA

TABLE 16 PARTS A - K.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<20	<20	<20	<20
1.5 HOURS	130	150	140	140
2.5 HOURS	190	225	200	205
3.5 HOURS	895	760	905	855
4.5 HOURS	1200	1070	1305	1200
5.5 HOURS	925	1130	1150	1085
6.5 HOURS	1385	1470	1270	1380
7.5 HOURS	2500	2305	2295	2370
8.5 HOURS	2610	2635	2540	2595

TABLE 19A: ACCUMULATION OF GENE 1 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<35	<35	<35	<35
1.5 HOURS	450	465	470	460
2.5 HOURS	911	985	870	920
3.5 HOURS	1100	1125	1070	1100
4.5 HOURS	1155	1205	1170	1175
5.5 HOURS	1370	1450	1270	1365
6.5 HOURS	1640	1550	1610	1600
7.5 HOURS	1670	1620	1550	1615
8.5 HOURS	1710	1780	1810	1765

TABLE 19B: ACCUMULATION OF GENE 2 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
[illegible]	<20	<20	<20	<20
1.5 HOURS	195	210	180	195
2.5 HOURS	330	315	285	315
3.5 HOURS	345	360	310	340
4.5 HOURS	330	375	345	345
5.5 HOURS	390	410	450	415
6.5 HOURS	990	1040	1145	1025
7.5 HOURS	925	1080	1160	1080
8.5 HOURS	1270	1480	1370	1365

TABLE 19(i) ACCUMULATION OF GENE 3 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
[illegible]	<20	<20	<20	<20
1.5 HOURS	70	65	80	70
2.5 HOURS	180	1\15	190	180
3.5 HOURS	380	2'/5	400	340
4.5 HOURS	420	385	480	428
5.5 HOURS	810	480	880	810
6.5 HOURS	790	750	800	780
7.5 HOURS	1480	1070	1200	1240
8.5 HOURS	2660	2220	2870	2520

TABLE 19(ii) ACCUMULATION OF GENE 4 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<10	<10	<10	<10
1.5 HOURS	15	20	10	15
2.5 HOURS	125	130	110	120
3.5 HOURS	175	155	160	165
4.5 HOURS	150	145	140	145
5.5 HOURS	135	160	155	150
6.5 HOURS	380	410	445	415
7.5 HOURS	545	550	550	550
8.5 HOURS	610	565	580	585

TABLE 16E: ACCUMULATION OF GENE 6 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<50	<50	<50	<50
1.5 HOURS	160	155	195	170
2.5 HOURS	345	405	410	385
3.5 HOURS	425	390	410	410
4.5 HOURS	550	550	575	505
5.5 HOURS	730	690	710	725
6.5 HOURS	1550	1490	1470	1515
7.5 HOURS	1050	990	1120	1050
8.5 HOURS	1120	1145	1155	1140

TABLE 16F: ACCUMULATION OF GENE 6 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / MOLECULES PER CELL			
	1	2	3	MEAN
UNINFECTED	<40	<40	<40	<40
1.5 HOURS	<40	<40	<40	<40
2.5 HOURS	75	85	80	80
3.5 HOURS	150	130	160	145
4.5 HOURS	210	200	180	195
5.5 HOURS	280	240	235	245
6.5 HOURS	360	360	410	385
7.5 HOURS	470	460	485	470
8.5 HOURS	545	560	575	560

TABLE 16G: ACCUMULATION OF GENE 7 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / MOLECULES PER CELL			
	1	2	3	MEAN
UNINFECTED	<20	<20	<20	<20
1.5 HOURS	200	170	185	185
2.5 HOURS	260	230	245	245
3.5 HOURS	270	240	265	260
4.5 HOURS	300	280	310	300
5.5 HOURS	390	310	380	380
6.5 HOURS	525	550	535	535
7.5 HOURS	580	620	630	615
8.5 HOURS	710	620	690	675

TABLE 16H: ACCUMULATION OF GENE 8 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / MOLECULES PER CELL			
	1	2	3	MEAN
UNINFECTED	<35	<35	<35	<35
1.5 HOURS	500	480	535	510
2.5 HOURS	570	535	550	550
3.5 HOURS	1050	885	810	850
4.5 HOURS	1120	995	1050	1055
5.5 HOURS	1070	1210	1170	1150
6.5 HOURS	2140	2120	1940	2065
7.5 HOURS	2140	2320	2310	2255
8.5 HOURS	2480	2360	2480	2440

TABLE 16b ACCUMULATION OF GENE 9 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / MOLECULES PER CELL			
	1	2	3	MEAN
UNINFECTED	<50	<50	<50	<50
1.5 HOURS	<50	<50	<50	<50
2.5 HOURS	50	55	50	50
3.5 HOURS	95	110	85	95
4.5 HOURS	100	85	105	100
5.5 HOURS	150	140	135	145
6.5 HOURS	200	190	215	200
7.5 HOURS	225	215	195	210
8.5 HOURS	310	305	290	300

TABLE 16c ACCUMULATION OF GENE 10 MINUS-SENSE RNA.

TIME POST-INFECTION	REPLICATE ASSAYS / 1	2	MOLECULES PER CELL 3	MEAN
UNINFECTED	<34	<34	<34	<34
1.5 HOURS	65	70	80	70
2.5 HOURS	235	260	240	245
3.5 HOURS	410	405	390	400
4.5 HOURS	355	370	360	360
5.5 HOURS	400	415	415	410
6.5 HOURS	380	420	425	410
7.5 HOURS	1480	1235	1360	1360
8.5 HOURS	1155	1255	1175	1195

TABLE 19K: ACCUMULATION OF GENE 11 MINUS-SENSE RNA.

TABLE 17: A SUMMARY OF THE ROTAVIRUS PROTEINS

The two main groups working with the UKtc and SA11 rotavirus strains (McCrae, Warwick, U.K. and Estes, Houston, U.S.A.) fail to agree on the location of several of the Rotavirus polypeptides. For clarity, a table is given listing the location (where known) of each polypeptide for these two rotavirus strains. This Table should be viewed in conjunction with Figure 2 on page 19.

Since the submission of this manuscript, Liu et al (1988) have been able to identify a protein product for genome segment 3 of the SA11 strain. They propose in this most recent publication that this product be termed VP3, and the gene 4 product previously known as VP3 be renamed VP4. The SA11 strain protein nomenclature now agrees with that of the UKtc strain after McCrae and Faulkner-Valle (1981).

GENE	PROTEIN PRODUCT IN UKtc	PROTEIN PRODUCT IN SA11
1	VP1 structural, inner shell	VP1 structural, inner shell
2	VP2 structural, inner shell	VP2 structural, inner shell
3	VP3 structural, inner shell	**VP3 structural, inner shell
4	VP4 structural, outer shell	*VP4 structural, outer shell
5	VP5 structural, location uncertain	NS53 nonstructural
6	VP6 structural, inner shell	VP6 structural, inner shell
7	VP8 probably nonstructural	NS34 nonstructural
8	VP7 structural, outer shell	NS35 nonstructural
9	VP9 nonstructural	VP7 structural, outer shell
10	VP10 nonstructural	NS20 nonstructural
11	VP11 nature not yet determined	VP9 structural, outer shell

* formerly VP3 ** Newly identified, Liu et al 1988

TABLE 17: A SUMMARY OF THE ROTAVIRUS PROTEINS.

REFERENCES.

REFERENCES

ACS G, KLETT H, SCHONBERG M, CHRISTMAN J, LEVIN DH, SILVERSTEIN SC (1971): Mechanism of Reovirus ds-RNA synthesis in vivo and in vitro. JOURNAL OF VIROLOGY 8 684-689.

ADAMS WR, KRAFT LM (1963): Epizootic diarrhea of infant mice: Identification of the etiologic agent. SCIENCE 141 359-360.

AHMED R, GRAHAM AF (1977): Persistant infection in L-cells with ts mutants of Reovirus. JOURNAL OF VIROLOGY 23 250-262.

ALBERT MJ, UNICOMB LE, BISHOP RF (1987): Cultivation and characterisation of Human Rotaviruses with "super-short" RNA patterns. JOURNAL OF CLINICAL MICROBIOLOGY 25 183-185.

ALLEN AM, DESSELBERGER U (1985): Reassortment of human Rotaviruses carrying rearranged genomes with bovine Rotavirus. JOURNAL OF GENERAL VIROLOGY 66 2703-2714.

ALMEIDA JD, HULL T, BANATVALA JE, TOTTERDELL BM, CHRYSTIE IL (1978): The effect of trypsin on the growth of Rotavirus. JOURNAL OF GENERAL VIROLOGY 40 213-218.

ALTENBURG BC, GRAHAM DY, ESTES MK (1980): Ultrastructural study of Rotavirus replication in cultured cells. JOURNAL OF GENERAL VIROLOGY 48 75-85.

ALWINE JC, KEMP DJ, STARK GR (1979): Method for detection of specific RNAs in agarose gels by transfer to Diazobenzylmethyl paper and hybridisation with DNA probes. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES. 74 5350-5354.

ANDERSON EL, BELSHE RB, BARTRAM J, CROOKSHANKS-NEWMAN F, CHANOCK RM, KAPIKIAN AZ (1986): Evaluation of rhesus rotavirus vaccine (MMU 18006) in infants and young children. JOURNAL OF INFECTIOUS DISEASES 153 823-831.

ARGARWAL A (1979): A cure for a killer-: but how to administer it. NATURE 278 389-381.

ASTELL C, SILVERSTEIN SC, LEVIN DH, ACS G (1972): Regulation of the Reovirus RNA transcriptase by a viral capsomere protein. VIROLOGY 48 648-654.

BABIUK LA, MOHAMMED K, SPENCE L, FAUVEL M, PETRO R (1977): Rotavirus isolation and cultivation in the presence of trypsin. JOURNAL OF CLINICAL MICROBIOLOGY 6 610-617.

BANFIELD WG, KASNIC G, BLACKWELL JK (1968): Further observations on the virus of epizootic diarrhoea of infant mice: An electron microscopic study. VIROLOGY 36 411-421.

BARTZ CR, CONKLIN RH, TUNSTALL CB, STEELE JH (1980): Prevention of murine Rotavirus infection with chicken egg yolk immunoglobulins. JOURNAL OF INFECTIOUS DISEASES 142 439-441.

BAYBUTT HN, McCRAE MA (1984): The molecular biology of Rotaviruses VII. Detailed structural analysis of Gene 10 of bovine Rotavirus. VIRUS RESEARCH 1 533-541.

BEARDS GM, PILFOLD JN, THOULESS ME, FLEWETT TH (1980): Rotavirus serotypes by serum neutralisation. JOURNAL OF MEDICAL VIROLOGY 5 231-237.

BELLAMY AR, JOKLIK WK (1967): Studies on the A-rich RNA of Reovirus RNA 1. Characterisation of Reovirus genome RNA. JOURNAL OF MOLECULAR BIOLOGY 28 1-18.

BELLAMY AR, NICHOLS JL, JOKLIK WK (1972): Nucleotide sequences of Reovirus oligonucleotides. Evidence for abortive RNA synthesis during virus maturation. NATURE (LONDON) NEW BIOLOGY 239 49-51.

BERK AJ, SHARP PA (1977): Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 nuclease digested hybrids. CELL 12 721-732.

BERNSTEIN JM, HRUSKA JF (1981): Characterisation of RNA polymerase products of Nebraska calf diarrhea virus and SA11 Rotavirus. JOURNAL OF VIROLOGY 37 1071-1074.

BERNSTEIN DI, ZIEGLER JM, WARD RL (1986): Rotavirus fecal IgA antibody response in adults challenged with human Rotavirus. JOURNAL OF MEDICAL VIROLOGY 20 297-304.

BESSELAAR TG, ROSENBLATT A, KIDD AH (1986): Atypical Rotavirus from South African neonates. ARCHIVES OF VIROLOGY 87 327-330.

BICAN P, COHEN J, CHARPILIENNE A, SCHERRER R (1982): Purification and characterisation of Bovine Rotavirus cores. JOURNAL OF VIROLOGY 43 1113-1117.

BIRBOIM HC, DOLY J (1979): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. NUCLEIC ACID RESEARCH 7 1513-1520.

BIRCHMEIER C, SCHUMPERLI D, SCONZO G, BIRNSTEIL ML (1984): 3' editing of mRNAs: Sequence requirements and involvement of a nucleotide RNA in maturation of histone mRNA precursors. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE 81 1057-1061.

BISHAI FR, SPENCE L, GOODWIN D, PERTO R (1979): Use of antisera against bovine (NCDV) and Simian (SA11) Rotaviruses in ELISA to detect different types of Human Rotavirus. CANADIAN JOURNAL OF MICROBIOLOGY 25 1118-1124.

BISHOP RF, DAVIDSON GP, HOLMES IH, RUCK BJ (1974): Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. LANCET 149-151.

BISHOP RF, CAMEROM DJS, VEENSTRA AA, BARNES GL (1979): Diarrhea and Rotavirus infection associated with different regimens for post-natal care of newborn babies. JOURNAL OF CLINICAL MICROBIOLOGY 9 525-529.

BISHOP RF (1984): Rotavirus in perspective. A personal view. AUSTRALIAN PEDIATRIC JOURNAL 20 8-12.

BOHL EH (1979): Rotaviral diarrhoea in pigs: Breif review. JOURNAL OF THE AMERICAN VETERINARY ASSOCIATION. 174 613-615.

BORSA J, GRAHAM AF (1968): Reovirus RNA polymerase activity in purified virions. BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 33 896-901.

BORSA J, GROVER J, CHAPMAN JD (1970): Presence of nucleoside triphosphate phosphohydrolase activity in purified virions of Reovirus. JOURNAL OF VIROLOGY 6 295-300.

BORSA J, COPPS TP, SARGENT MD, LONG DG, CHAPMAN JD (1973): New intermediate sub-viral particles in the in vitro uncoating of Reovirus virions by chymotrypsin. JOURNAL OF VIROLOGY 11 552-564.

BORSA J, LONG DG, COPPS TP, SARGENT MD, CHAPMAN JD (1974a): Reovirus transcriptase activation in vitro: Further studies on the facilitation phenomenon. INTERVIROLOGY 3 15-35.

BORSA J, LONG DG, SARGENT MD, COPPS TP, CHAPMAN JD (1974b): Reovirus transcriptase activation in vitro: Involvement of an endogenous uncoating activity in the second stage of the process. INTERVIROLOGY 4 171-188.

BOTH GW, SIEGMAN LJ, BELLAMY AR, ATKINSON PH (1983): Coding assignments and nucleotide sequence of Simian Rotavirus SA11 gene segment 10: Location of glycosylation sites suggests that the signal peptide is not cleaved. JOURNAL OF GENERAL VIROLOGY 48 335-339.

BOYLE JF, HOLMES KV (1986): RNA binding proteins of bovine Rotavirus. JOURNAL OF VIROLOGY 58 561-568.

BRADE L, SCHMIDT WAK (1979): Simian Rotavirus (SA11) in serodiagnosis of human Rotavirus infections. MEDICAL MICROBIOLOGY AND IMMUNOLOGY 167 55-60.

BRAHIC M, HAASE AT (1978): Detection of viral sequences of low reiteration frequency by in situ hybridisation. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE 75 6125-6129.

BRIDGER JC, WOODE GN (1976): Characterisation of two particle types of calf Rotavirus. JOURNAL OF GENERAL VIROLOGY 31 245-250.

BRIDGER JC (1978): Location of type specific antigens in calf Rotavirus. JOURNAL OF CLINICAL MICROBIOLOGY 8 625-628.

BRIDGER JC (1980): Detection by electron microscopy of Caliciviruses, Astroviruses, and Rotavirus-like particles in the faeces of piglets with Diarrhea. VETERINARY RECORD 107 532-533.

BRIDGER JC, CLARKE IN, McCRAE MA (1982): Characterisation of an antigenically distinct porcine Rotavirus. INFECTION AND IMMUNITY 35 1058-1062.

BRIDGER JC, PEDLEY S, McCRAE MA (1986): Group C Rotaviruses in humans. JOURNAL OF CLINICAL MICROBIOLOGY 23 760-763.

BRIDGER JC (1987): Novel Rotaviruses in animals and man. In "Novel diarrhoea viruses". Ciba foundation symposium 128 published by Wiley and Sons 1987 p5-23.

BROWN DWG, BEARDS G K, CHEN GM, FLEWETT TH (1987): Prevalence of antibody to Group B (Atypical) Rotavirus in humans and animals. JOURNAL OF CLINICAL MICROBIOLOGY 25 316-319.

BRUSSOW H, MARC-MARTIN S, EICHHORN W, SLDOTI J, FRYDER V (1987): Characterisation of a second bovine Rotavirus serotype. ARCHIVES OF VIROLOGY 94 29-41.

BRYDEN AS, DAVIES HA, HADLEY RE, FLEWETT TH, MORRIS CA, OLIVER P (1975): Rotavirus enteritis in the West-Midlands during 1974. LANCET II 241.

BUTLER ET, CHAMBERLAIN MJ (1982): Bacteriophage Sp6 specific RNA polymerase. JOURNAL OF BIOLOGICAL CHEMISTRY 257 5772-5778.

CAMERON DS, BISHOP RF, VEENSTRA AA, BARNES GL (1978): Noncultivable viruses and neonatal diarhea; Fifteen month survey in a newborn special care nursery. JOURNAL OF CLINICAL MICROBIOLOGY 8 93-98.

CAMPBELL JL, RICHARDSON CC, STUDIER FW (1982): Genetic recombination and complementation between bacteriphage T7 and cloned fragments of T7 DNA. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES 78 2276-2280.

CARPIO MM, BABUIK LA, MISRA V, BLUMENTHAL RM (1981): Bovine Rotavirus-cell interactions: Effect of virus infection on cellular integrity and macromolecular synthesis. VIROLOGY 114 86-97.

CHAN WK, PANARANDA ME, CRAWFORD SE, ESTES MK: (1986): Two glycoproteins are produced from the Rotavirus Neutralisation gene. VIROLOGY 151 243-252.

CHANDA PK, BANERJEE AK (1981): Identification of promotor proximal oligonucleotides and a unique oligonucleotide pppGpC from in vitro transcription products of Vesicular Stomatitis virus. JOURNAL OF VIROLOGY 39 93-103.

CHANG CT, ZWEERINK HJ (1971): Fate of parental Reovirions in infected cells. VIROLOGY 46 544-555.

CHASEY D (1977): Different particle types in tissue culture and intestinal epithelium infected with Rotavirus. JOURNAL OF GENERAL VIROLOGY 37 443-451.

CHASEY D (1980): Investigation of immunoperoxidase-labelled Rotavirus in tissue culture by light and electron microscopy. JOURNAL OF GENERAL VIROLOGY 50 195-200.

CHASEY D, BANKS J (1984): The commonest Rotavirus from neonatal lamb diarrhoea in England and Wales have atypical electropherotypes. VETERINARY RECORD 115 326-327.

CHASEY D, DAVIES P (1984): Atypical Rotaviruses in pigs and cattle. VETERINARY RECORD. 114 16-17.

CHASEY D, BANKS J (1986): Replication of Atypical ovine Rotavirus in small intestine and cell culture. JOURNAL OF GENERAL VIROLOGY 67 567-576.

CHASEY D, BRIDGER JC, McCRAE MA (1986): A new type of Atypical Rotavirus in pigs. ARCHIVES OF VIROLOGY 89 235-243.

CHEEVER FS, MUELLER JH (1947): Epidemic diarrheal disease of suckling mice I. Manifestations, epidemiology ana attempts to transmit the disease. JOURNAL OF EXPERIMENTAL MEDICINE. 85 405-416.

CHEN G, HUNG T, BRIDGER JC, McCRAE MA (1985): Chinese adult Rotavirus is a group B Rotavirus. LANCET II 1123-1124.

CHIBA Y, MIYAZAKI C, MAKINO Y, MATANDA LN, KIBUEA A, LACHENGA EO, TUREI PM (1984): Rotavirus infection of young children in two districts of Kenya from 1982 to 1983 as analysed by electrophoresos of Genomic RNA. JOURNAL OF CLINICAL MICROBIOLOGY 19 579-582.

CHRYSTIE IL, TOTTERDELL BM, BANATVALA JE (1978): Asymptomatic endemic Rotavirus infections in the newborn. LANCET I 1176.

CLARK HF, OFFIT PA, DOLAN KT, TEZZA A, GOGALIN K, TWIST EM, PLOTKIN SA (1986): Response of adult human volunteers to oral administration of bovine and human/bovine reassortant Rotaviruses. VACCINE 4 25-31.

CLARK SM, SPENDLOVE RS, BARNETT BB (1980): Role of two particle types in Bovine Rotavirus morphogenesis. JOURNAL OF VIROLOGY 34 272-276.

CLARKE IN, McCRAE MA (1981): A rapid and sensitive method for analysing the genome profiles of field isolates of Rotavirus. JOURNAL OF VIROLOGICAL METHODS 2 203-209.

CLARKE IN, McCRAE MA (1981a): A sensitive method for the production of diagnostic fingerprints of the genome segments of field isolates of Rotavirus. JOURNAL OF VIROLOGICAL METHODS 3 261-269.

CLARKE IN, McCRAE MA (1982): Structural analysis of electrophoretic variation of the genome profiles of Rotavirus field isolates. INFECTION AND IMMUNITY 36 492-497.

CLARKE IN, McCRAE MA (1983): The molecular biology of Rotaviruses VI: RNA species-specific terminal conservation in Rotaviruses. JOURNAL OF GENERAL VIROLOGY 64 1877-1884.

COHEN J (1977): Ribonucleic acid polymerase activity associated with purified calf Rotavirus. JOURNAL OF GENERAL VIROLOGY 36 395-402.

COHEN J, DOBOS P (1979): Cell-free transcription and translation of Rotavirus RNA. BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 88 791-796.

COHEN J, LAPORTE J, CHARPILIENNE A, SCHERRER R (1979): Activation of Rotavirus RNA polymerase by calcium chelation. ARCHIVES OF VIROLOGY 60 177-186.

COLLINS PK, FULLER FJ, MARCUS PI, HIGHTOWER LE, BALL LA (1982): Synthesis and processing of Sindbis virus non-structural proteins in vitro. VIROLOGY 118 363-379.

CORTHIER G, FRANZ JR (1981): Detection of anti-Rotavirus immunoglobulins A,G and M in swine colostrum, milk and feces by enzyme-linked immunosorbent assays. INFECTION AND IMMUNITY 31 833-836.

COX KH, DELEON DV, ANGERER LM, ANGERER RC(1984): Detection of messenger RNAs in sea urchin embryos by in situ hybridisation using asymetric RNA probes. DEVELOPMENTAL BIOLOGY 101 485-502.

CRAWFORD NM, BALTIMORE D (1983): Genome linked protein Vpg of poliovirus present as Vpg and VpgpUp in Poliovirus infected cells. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA. 80 7452-7455.

CRUICKSHANK JG, AXTON JHM, NEBSTER OF (1974): Viruses in gastroenteritis. LANCET I 1353.

CUBITT WD, HOLZEL M (1980): An outbreak of Rotavirus infection in a long-stay ward of a geriatric hospital. JOURNAL OF CLINICAL PATHOLOGY 33 306-308.

CUKOR G, BLACKLOW NR, CAPOSSA FE, PANJVANI ZFK, BEDNAREK F (1979): Persistence of antibodies to Rotavirus in human milk. JOURNAL OF CLINICAL MICROBIOLOGY 9 93-96.

COULSON BS, FOLER KJ, BISHOP RF, COTTON RG (1985): Neutralising monoclonal antibodies to human Rotavirus and indications of antigenic drift among strains from neonates. JOURNAL OF VIROLOGY 54 14-20.

DAVANLOO P, ROSENBERG AH, DONN JJ, STUDIER FW (1984): Cloning and expression of the gene for bacteriophage T7 RNA polymerase. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 81 2035-2039.

DAVIDSON GP, BISHOP RF, TOWNLEY RRW, HOLMES IH, RUCK BJ (1975): Importance of a new virus in acute sporadic enteritis in children. LANCET I 242-246.

DAVIDSON GP, GALL DG, PETRIC M, BUTLER DG, HAMILTON JR (1977): Human Rotavirus enteritis induced in conventional piglets: intestinal structure and transport. JOURNAL OF CLINICAL INVESTIGATION 60 1402-1409.

DAVIDSON GP, BARNES GL (1979): Structural and functional abnormalities of the small intestine in infants and young children with Rotavirus enteritis. ACTA PEDIATRICA SCANDINAVICA (Stockholm) 68 181-186.

DE MOL P, ZISSIS G, MUTWEWINGABO A, ANDRE FE (1986): Failure of live attenuated oral Rotavirus vaccine. LANCET I 108.

DE ZOYSA I, FEACHAM RG (1986): Interventions for the control of diarhoeal diseases among young children: Rotavirus and Cholera immunization. WORLD HEALTH ORGANISATION BULLETIN 63 569-583.

DIAZ M, BARSACCHI-PILONE G, MAHON K, GALL J (1981): Transcripts from both strands of a satellite DNA occur on Lampbrush chromosome loops of the newt Notophthalmus. CELL 24 649-659.

DOLAN KT, TWIST EM, HORTON-SLIGHT P, FORRER C, BELL LM, PLOTKIN SA, CLARK HF (1985): Epidemiology of Rotavirus electropherotypes determined by a simplified diagnostic technique with RNA analysis. JOURNAL OF CLINICAL MICROBIOLOGY 21 753-758.

DROZDOV SG, SHEKOYAN LA, KOROLEV MB, ANDZHAPARIDZE AG(1978): Human Rotavirus in cell culture ; Isolation and passaging. VORP VIRUSOL 4 389-392.

DRYNA D, FIELDS BN (1982): Activation and characterisation of the Reovirus transcriptase; Genetic analysis. JOURNAL OF VIROLOGY 41 110-118.

DUNN JJ, STUDIER FW (1983): Complete nucleotide sequence of Bacteriophage T7 DNA and the locations of T7 genetic elements. JOURNAL OF MOLECULAR BIOLOGY 166 477-535.

DUFFY LC, BYERS TE, RIEPENHOFF-TALTY M, LA SCOLEA LJ, ZIELEZNY M, OGRA PL (1986): The effects of infant feeding on Rotavirus-induced gastroenteritis: A prospective study. AMERICAN JOURNAL OF PUBLIC HEALTH 76 259-263.

DYALL-SMITH ML, HOLMES IH (1981): Gene coding assignment of Rotavirus Double-stranded RNA. Segments 10 and 11. JOURNAL OF VIROLOGY 39 1099-1103.

ELIAS MM (1977): Separation and infectivity of two particle types of human Rotavirus. JOURNAL OF GENERAL VIROLOGY 37 191-194.

EMERSON SU (1982): Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome. CELL 31 635-642.

EMERSON SU (1985): Rhabdoviruses, Chapter 48 in "Virology" Edited by FIELDS BN. Published by Raven press, New York 1119-1132.

ERICSON BL, GRAHAM DY, MASIN BB, ESTES MK (1982): Identification, synthesis and modifications of Simian Rotavirus SA11 polypeptides in infected cells. JOURNAL OF VIROLOGY 42 825-839.

ERNST H, SHATKIN AJ (1986): Reovirus haemagglutinin mRNA codes for two polypeptides in overlapping reading frames. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 82 48-52.

ESPARZA J, GIL F (1978): A study on the ultrastructure of Human Rotavirus. VIROLOGY 25 141-150.

ESPARZA J, GORZIGLIA M, GIL F, RONER H (1980): Multiplication of human Rotavirus in cultured cells: An electron microscopic study. JOURNAL OF GENERAL VIROLOGY 47 461-472.

ESPEJO RT, AVENDANO LF, MUNOZ O, ROMERO P, ETERNOD JG, LOPEZ S, MONCAYA J (1980a): Comparison of human Rotaviruses isolated in Mexico City and in Santiago, Chile, by electrophoretic migration of their double-stranded Ribonucleic acid genome segments. INFECTION AND IMMUNITY 30 342-348.

ESPEJO RT, MUNOZ O, SERAFIN F, ROMERO P (1980b): Shift in the prevalent human Rotavirus detected by Ribonucleic acid segment differences. INFECTION AND IMMUNITY 27 351-354.

ESPEJO RT, LOPEZ S, ARIAS C (1981): Structural polypeptides of Simian Rotavirus SA11 and the effect of trypsin. JOURNAL OF VIROLOGY 37 156-160.

ESTES MK, GRAHAM DY, GERBA CP, SMITH EM (1979): Simian Rotavirus SA11 replication in cell culture. JOURNAL OF VIROLOGY 31 810-815.

ESTES MK, GRAHAM DY, MASON BA (1981): Proteolytic enhancement of Rotavirus infectivity: Molecular mechanisms. JOURNAL OF VIROLOGY 39 879-888.

ESTES MK, OBIJESKI JF, PALMER EL (1983): Rotaviruses :A reveiw. CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY 105 123-184.

ESTES MK, CRAWFORD SE, PENARANDA ME, PETRIE BL, BURNS JW, CHAN W-K, ERICSON B, SMITH GE, SUMMERS MD (1987): Synthesis and immunogenicity of the Rotavirus major capsid antigen using a baculovirus expression system. JOURNAL OF VIROLOGY 61 1488-1494.

FAULKNER-VALLE GP, CLAYTON AV, McCRAE MA (1982): Molecular biology of Rotaviruses III: Isolation and characterisation of ts mutants of Bovine Rotavirus. JOURNAL OF VIROLOGY 42 669-677.

FAULKNER-VALLE GP, LEWIS J, PEDLEY S, McCRAE MA (1983): Isolation and characterisation of ts mutants of bovine Rotavirus. In DS RNA VIRUSES. Edited by Compans RW and Bishop DHL. (Elseivier publishing co.) 303-312.

FINBERG R, WEINER HL, FIELDS BN, BENACERRAF B, BURAKOFF SJ (1979): Generation of cytolytic T lymphocytes after virus infection: Role of the S1 Gene. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 74 442-446.

FLEWETT TH, BRYDEN AS, DAVIES HA (1973): Virus particles in gastroenteritis. LANCET II 1497.

FLEWETT TH, BRYDEN AS, DAVIES H, WOODE GN, BRIDGER JC, DERRICK JM (1974): Relation between viruses from acute gastroenteritis of children and newborn calves. LANCET II 61-63.

FLEWETT TH (1977): Acute non-bacterial infectious gastroenteritis- an essay in comparative virology. In RECENT ADVANCES IN CLINICAL VIROLOGY. 1 Edited by WATERSON AP Published by Churchill Livingstone, Edinburgh 151-169.

FLEWETT TH, THOULESS ME, PILFORD JN, BRYDEN AS, CANDEIAS JAN (1978): More serotypes of human Rotavirus. LANCET II 632.

FLEWETT TH, WOODE GN (1978): The Rotaviruses: Brief review. ARCHIVES OF VIROLOGY 57 1-23.

FLEWETT TH (1986): Rotavirus vaccines: achievements and prospects. ARCHIVES OF DISEASES IN CHILDHOOD 61 211-212.

FOLLETT EAC, DESSELBERGER U (1983a): Co-circulation of different Rotavirus strains in a local outbreak of infantile gastroenteritis. Monitoring by rapid and sensitive nucleic acid analysis and by oligonucleotide mapping. In DS RNA VIRUSES. Edited by Compans RW and Bishop DHL. (Elseivier publishing co.) 331-342.

FOLLETT EAC, DESSELBERGER U (1983b): Co-circulation of different Rotavirus strains in a local outbreak of infantile gastroenteritis: Monitoring by rapid and sensitive nucleic acid analysis. JOURNAL OF MEDICAL VIROLOGY 11 39-52.

FOLLETT EAC, SANDERS RC, BEARDS GM, HUNDLEY F, DESSELBERGER U (1984): Molecular epidemiology of human Rotaviruses. Analysis of outbreaks of acute gastroenteritis in Glasgow and the west of Scotland 1981/82 and 1982/83. JOURNAL OF HYGEINE 92 209-222.

FONTANA A, WEINER HL (1980): Interaction of Reovirus with cell surface receptors II: Generation of supressor T cells by the haemagglutinin of Reovirus type 3. JOURNAL OF IMMUNOLOGY 125 2660-2664.

FONTEYNE J, ZISSIS G, LAMBERT JP (1978): Recurrent Rotavirus gastroenteritis. LANCET I 983.

FURUICHI Y, MORGAN M, MITHUKRISHNAN S, SHATKIN AJ (1975): Reovirus messenger RNA contains a methylated, blocked 5' terminal structure: m7G(5')ppp(5')GmpCp. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 72 362-366.

GAILLARD RK, JOKLIK WK (1985): The relative translation efficiencies of Reovirus messenger RNAs. VIROLOGY 147 336-348.

GAUL SK, SIMPSON TF, WOODE GN, FULTON RW (1982): Antigenic relationships among some animal Rotaviruses, virus neutralisation and cross protection in pigiets. JOURNAL OF CLINICAL MICROBIOLOGY 15 495-503.

GILLIES S, BULLIVANT, BELLAMY AR (1971): Viral RNA polymerase: Electron microscopy of Reovirus reaction cores. SCIENCE 174 694-696.

GOMATOS PJ, TAMM I (1963): Animal and plant viruses with double helical RNA. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 50 878-885.

GOMATOS PJ (1967): RNA synthesis in Reovirus infected L929 mouse fibroblasts. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 58 1798-1804

GOMBOLD JL, RAMIG RF (1987): Assignment of Simian Rotavirus SA11 temperature sensitive mutants Groups A, C, F and G to genome segments. VIROLOGY 161 463-473.

GOMEZ JA, BISCOTTO EL, BERCOVICH JA, GRUNSTEIN S (1986): Epidemiology of human Rotavirus in Argentina as determined by RNA genome electrophoresis. INTERVIROLOGY 26 174-180.

GORZIGLIA M, ESPARZA J (1981): Poly(A) polymerase activity in human Rotavirus. JOURNAL OF GENERAL VIROLOGY 53 357-362.

GORZIGLIA M, HOSHINO Y, BUCKLER-WHITE A, BLUMENTALS I, GLASS R, FLORES J, KAPIKIAN AZ, CHANOCK RM (1986): Conservation of amino acid sequence of VP8 and cleavage region of 84KDa outer capsid proteins among Rotavirus recovered from asymptomatic neonatal infection. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 83 7039-7043.

GRAHAM DY, ESTES MK (1980): Proteolytic enhancement of Rotavirus infectivity: Biological mechanaims. VIROLOGY 101 432-439.

GRAUBALLE PG, VESTERGAARD BF, MEYLING A, GENNER J (1981): Optimised Enzyme Linked Immunosorbent Assay for the detection of human and bovine Rotaviruses in stools: Comparison with Electron microscopy, Immunoelectrophoresis and Fluorescent antibody techniques. JOURNAL OF MEDICAL VIROLOGY 7 29-40.

GREEN MR, MANIATIS T, MELTON DA (1983): Human beta-globulin pre-mRNA syntnesised in vitro is accurately spliced in Xenopus oocyte nuclei. CELL 32 681-694.

GREENBERG HB, KALICA AR, WYATT RG, JONES RW, KAPIKIAN AZ, CHANOCK RM (1981): Rescue of non-cultivatable human Rotaviruses by gene reassortment during mixed infection with ts mutants of a cultivatable bovine Rotavirus. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 78 420-424.

GREENBERG HB, WYATT RG, KAPIKIAN AZ, KALICA AR, FLORES J. JONES R (1982): Rescue and serotypic characterisation of non-cultivatable human Rotaviruses by gene reassortment. INFECTION AND IMMUNITY 37 104-109.

GREENBERG HB, FLORES J, KALICA AR, WYATT RG, JONES RW (1983a): Gene coding assignments for growth restriction, neutralisation and subgroup specificities of the W and DS1 strains of human Rotavirus. JOURNAL OF GENERAL VIROLOGY 64 313-320.

GREENBERG H, KALIKA A, FLORES J, KAPIKIAN A, WYATT R, JONES R, VALDESUSO J (1983b): Gene-coding assignments for Rotavirus studied with genetic reassortants and monoclonal antibodies. In DS RNA VIRUSES. Edited by Compans RW and Bishop DHL. (Elsevier publishing co.) 289-302.

GREENBERG HB (1987): In discussion of: Novel Rotaviruses in animals and man. By BRIDGER JC in "Novel diarrhoea viruses". Ciba foundation symposium 128, published by Wiley and Sons 1987 p5-23.

GUERRIER-TAKADA C, ALTMAN S (1984): Catalytic activity of an RNA molecule prepared by transcription in-vitro. SCIENCE 223 285-286.

HALVORSRUD J, ORSTAVIC I (1980): An epidemic of Rotavirus associated gastroenteritis in a nursing home for the elderly. SCANDANAVIAN JOURNAL OF INFECTIOUS DISEASE 12 161-164.

HAMILTON JR, GALL DG, BUTLER DG, MIDDLETON PJ (1976): Viral gastroenteritis: Recent progress, remaining problems. in ACUTE DIARRHOEA IN CHILDHOOD - CIBA FOUNDATION SYMPOSIUM No 42. Edited by Elliot and Knight. Published by Elsevier/Excepta Medica 209-219.

HANECAK R, SEMLER BL, ANDERSON CW, WIMMER E (1982): Proteolytic processing of poliovirus polypeptides: Antibodies to a polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 79 3973-3977.

HARVEY JD,BELLAMY AR, EARNSHAW WC, SCHUTT C (1981): Biophysical studies of Reovirus Type 3 IV. Low angle X-ray diffraction studies. VIROLOGY 112 240-249.

HAYES EC, LEE PWK, MILLER SE, JOKLIK WK (1981): The interaction of a series of hybridoma IgGs with Reovirus particles; Demonstration that the core protein 12 is exposed on the particle surface. VIROLOGY 108 147-155.

HELMBERGER-JONES M, PATTON JT (1986): Characterisation of subviral particles in cells infected with Simian Rotavirus SA11. VIROLOGY 155 655-665.

HENDERSON DR, JOKLIK WK (1978): The mechanism of interferon induction by uv irradiated Reovirus. VIROLOGY 91 389-406.

HERNANDEZ N, KELLER W (1983): Splicing of in-vitro synthesised messenger RNA precursors in HeLa cell extracts. CELL 35 89-99.

HERRING AJ, INGLIS NF, OJEH CK, SNODGRASS DR, MENZIES JD (1982): Rapid diagnosis of Rotavirus infection by direct detection of viral nucleic acid in silver stained polyacrylamide gels. JOURNAL OF CLINICAL MICROBIOLOGY 16 473-477.

HESS RG, BACHMANN PA (1981): Distribution of antibodies to Rotavirus in serum and lacteal secretions of naturally infected swine and their suckling pigs. AMERICAN JOURNAL OF VETERINARY RESEARCH 42 1149-1152.

HJELT G, GRAUBALLE PC, PAERREGAARD A, NEILSEN OH, KRASILNIKOFF PA (1987): Protective effect of preexisting Rotavirus specific immunoglobulin A against naturally acquired Rotavirus infection in children. JOURNAL OF MEDICAL VIROLOGY 21 39-47.

HOLLAND J, SPINDLER K, HORODYSKI F, GRABAU E, NICHOL S, VAN de POL S (1982): Rapid evolution of RNA genomes. SCIENCE 215 1577-1585.

HOLMES IH, RODGER SM, SCHNAGL RD, RUCH BJ, GUST ID, BISHOP RF, BARNES RD (1976): Is lactase the receptor and uncoating enzyme for infantile enteritis(rota) viruses? LANCET I 1387.

HOLMES IH (1979): Viral gastroenteritis. PROGRESS IN MEDICAL VIROLGY 25 1-36.

HOLMES IH (1983): Rotaviruses. Chapter 8 in "The Reoviridae" Edited by Joklik WK. Published by Plenun press 359-423.

HOSHINO Y, BALDWIN CA, SCOTT FW (1981): Isolation and characterisation of feline Rotavirus. JOURNAL OF GENERAL VIROLOGY 54 313-319.

HOSHINO Y, SERENO MM, MIDTHUN K, FLORES J, KAPIKIAN AZ, CHANOCK RM (1985): Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralisation of Rotavirus infectivity. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 82 8701-8704.

HUISMANS H, JOKLIK WK (1976): Reovirus-coded polypeptides in infected cells: isolation of two native monomeric polypeptides with affinity for single-stranded and double-stranded RNA respectively. VIROLOGY 70 411-424.

HUNDLEY F, BIRYAHWAHO B, GOW M, DESSELBERGER U (1985): Genome rearrangements of bovine Rotavirus after serial passage at high multiplicity of infection. VIROLOGY 143 88-103.

HUNDLEY F, McINTYRE M, CLARKE B, BEARDS G, WOOD D, CHRYSTIE I, DESSELBERGER U (1987): Heterogeneity of Genome rearrangements in Rotavirus isolated from a chronically infected immunodeficient child. JOURNAL OF VIROLOGY 61 3365-3372.

HUNG T, CHEN GM, WANG C, CHOU Z, CHAO T, YE W,YAO H, MENG K (1983): Rotavirus-like agent in adult non-bacterial diarrhoea in China. LANCET II 1078-1079.

HUNG T, CHEN GM, WANG C, YAO H, FANG Z, CHAO T, CHOU Z, YE W, CHANG X, DEN S, LIONG X, CHANG W (1984): Waterborne outbreak of Rotavirus diarrhoea in adults in China caused by a novel Rotavirus. LANCET I 1139-1142.

HUNG T, FAN R, WANG C, CHEN GM, CHOU D, CHANG J, McCRAE M, WANG W, SE W, DAN R, NG MH (1985): Seroepidemiology of adult Rotavirus. LANCET II 325-326.

IVERSON LE, ROSE JK (1982): Sequential synthesis of 5' proximal vesicular stomatitis virus mRNA sequences. JOURNAL OF VIROLOGY 44 356-365.

JACOBS BL, SAMUEL CE (1985): Biosynthesis of Reovirus-specified polypeptides: The reovirus S1 mRNA encodes two primary translation products. VIROLOGY 143 63-74.

JOKLIK WK (1981): Structure and function of the Reovirus genome. MICROBIOLOGICAL REVEIWS 45 483-501.

KALICA AR, GARON CF, WYATT RG, MEBUS CA, VAN KIRK DH, CHANOCK RM, KAPIKIAN AZ (1976): Differentiation of human and calf Reovirus-like agents associated with diarrhoea using polyacrylamide gel-electrophoresis of RNA. VIROLOGY 74 86-92.

KALICA AR, GREENBERG HB, WYATT RG, FLORES J, SERENO MM, KAPIKIAN AZ, CHANOCK RM (1981a): Genes of human (strain WA) and bovine (strain UK) Rotaviruses that code for neutralisation and subgroup antigens. VIROLOGY 112 385-390.

KALICA AR, GREENBERG HB, ESPEJO RT, FLORES J, WYATT RG, KAPIKIAN AZ, CHANOCK RM (1981b): Distinctive ribonucleic acid patterns of human Rotavirus subgroups 1 and 2. INFECTION AND IMMUNITY 33 958-961.

KALICA AR, FLORES J, GREENBERG HB (1983): Identification of the Rotaviral gene that codes for haemaggiutinin and protease-enhanced plaque formation. VIROLOGY 125 194-205.

KANTHARIDIS P, DYALL-SMITH ML, HOLMES IH (1983): Completion of the gene coding assignments of SA11 Rotavirus: Gene products of segments 7,8 and 9. JOURNAL OF VIROLOGY 48 330-334.

KAPIKIAN AZ, KIM HW, WYATT RG, RODRIGUEZ WJ, CLINE WL, PARROTT RH, CHANOCK RM (1974): Reovirus like agent in stools: Association with infantile diarrhoea and development of serologic tests. SCIENCE 185 1049-1053.

KAPIKIAN AZ, FLORES J, HOSHINO Y, GLASS RI, MIDTHUN K, GORZIGLIA M, CHANOCK RM (1986): Rotavirus: The major etiological agent of severe infantile diarrhea may be controllable by a "Jennerian" approach to vaccination. JOURNAL OF INFECTIOUS DISEASES 153 815-822.

KAPULER AM, MENDELSOHN N, KLETT H, ACS G (1970): Four base specific 5' triphosphates in the subviral core of Reoviruses. NATURE 225 1209-1211.

KASSAVETIS GA, BUTLER ET, ROULLAND D, CHAMBERLIN MJ (1982): Bacteriophage SP6-specific RNA polymerase. JOURNAL OF BIOLOGICAL CHEMISTRY 257 5779-5788.

KIM HW, BRANDT CD, KAPIKIAN AZ, WYATT RG, ARROBIO JO, RODRIGUEZ WJ, CHANOCK RM, PARROTT RH (1977): Human Reovirus-like agent infection: Occurance in adult contacts of pediatric patients with gastroenteritis. JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION 238 404.

KITAMUIRA N, SEMLER B, ROTHBERG PG, LARSEN GR. ADLER CJ, DOMER AJ, EMINI EA, HANECAK R, LEE JJ, VAN DER WERF S, ANDERSON CW, WIMMER E (1981): Primary structure, gene organisation and polypeptide expression of poliovirus RNA. NATURE 291 547-553.

KRAINER AR, MANIATIS T, RUSKIN B, GREEN MR (1984): Normal and mutant human beta pre-mRNAs are faithfully and efficiently spliced in vitro. CELL 36 993-1005.

KREIG PA. MELTON DA (1984): Formation of the 3' end of histone messenger RNA by post-trasnscriptional processing. NATURE 308 203-206.

KRUG RM, BOULOY M, PLOTCH SJ (1981): The 5' ends of influenza viral messenger RNAs are donated by capped cellular RNAs. TRENDS IN BIOCHEMICAL SCIENCES JAN 7-10.

KRUGER K, GRABOWSKI PJ, ZANG AJ, SANDS J, GOLTSHLING DE, CECH TR (1982): Self-splicing RNA: Autoexcision and autocyclization of the ribosomal RNA intervening sequence of tetrahymena. CELL 31 147-157.

LAEMMLI UK (1970): Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. NATURE 227 680-683.

LAU RY, VAN ALSTYNE D, BERKMANS R, GRAHAM DY (1975): Synthesis of Reovirus specific polypeptides in cells pretreated with cycloheximide. JOURNAL OF VIROLOGY 16 470-478.

LEE PWK, HAYES EC, JOKLIK WK (1981a): Characterisation of anti-reovirus immunoglobulins secreted by cloned hybridoma cell lines. VIROLOGY 108 134-146.

LEE PWK, HAYES EC, JOKLIK WK (1981b): Protein sigma 1 is the Reovirus cell attachment protein. VIROLOGY 108 156-163.

LEECE JG, KING MW, MOCK R (1976): Reovirus-like agent associated with fatal diarrhoea in neonatal pigs. INFECTION AND IMMUNITY 281 816-825.

LIU S, BIRCH C, COUPLEPIS A, GUST I (1984): Immunofocus assay for detection and quantitation of human Rotavirus. JOURNAL OF CLINICAL MICROBIOLOGY 20 347-350.

LOH PC, SHATKIN AJ (1968): Structural proteins of Reovirus. JOURNAL OF VIROLOGY 106 282-286.

LOPEZ S, ARIAS CF, BELL JR, STRAUSS JH, ESPEJO RT (1985): Primary structure of the cleavage site associated with trypsin enhancement of Rotavirus SA11 infectivity. VIROLOGY 144 11-19.

LOPEZ S, ARIAS C, MENDEZ E, ESPEJO RT (1986): Conservation in Rotaviruses of the protein region containing the two sites associated with trypsin enhancement of infectivity. VIROLOGY 154 224-227.

LYCKE E, BLOMBERG J, BERG G, ERICKSOON A, MADSEN L (1978): Epidemic acute diarrhoea in adults associated with infantile gastroenteritis. LANCET II 124.

MALHERBE HH, STRICKLAND-CHOMLEY M(1967): Simian virus SA11 and the related "O" agent. ARCHIV FUR DIE GESAMTE VIRUSFORCH 22 235-245.

MANIATIS T, FRITSCH EF, SAMBROOK J (1982): Molecular cloning, a laboratory manual. Published by Cold Spring Harbor Laboratory 1982.

MANLEY J (1983): Accurate and specific polyadenlyation of mRNA precursors in a soluble whole-cell lysate. CELL 33 595-605.

MASON BB, GRAHAM DY, ESTES MK (1980): In vitro transcription and translation of Simian Rotavirus SA11 gene products. JOURNAL OF VIROLOGY 33 1111-1121.

MATSUNO S, ONOUYE S, KONO R (1977): Antigenic relationship between human and bovine Rotavirus as determined by neutralisation, immune adherence haemagglutinin and complement fixation tests. INFECTION AND IMMUNITY 17 661-662.

MATSUNO S, HASEGAWA A, KALICA AR, KONO R (1980): Isolation of a recombinant between simian and bovine Rotaviruses. JOURNAL OF GENERAL VIROLOGY 48 253-256.

MATSUNO S, HASEGAWA A, MUKOYAMA A, INNOYE S (1985): A candidate for a new serotype of human Rotavirus. JOURNAL OF VIROLOGY 54 623-625.

McALLISTER WT, MORRIS C, ROSENBERG AH, STUDIER FW (1981): Utilisation of Bacteriophage T7 late promotors in recombinant plasmids during infection. JOURNAL OF MOLECULAR BIOLOGY 153 527-534.

McCRAE MA, JOKLIK WK (1978): The nature of the polypeptide encoded by each of the 10 double stranded RNA segments of Reovirus type 3. VIROLOGY 89 578-593.

McCRAE MA, FAULKNER-VALLE GP (1981): The molecular biology of Rotaviruses I. Characterisation of basic growth parameters and pattern of macromolecular synthesis. JOURNAL OF VIROLOGY 39 490-496.

McCRAE MA, McCORQUODALE JG (1982a): The molecular biology of Rotavriuses II. Identification of the protein-coding assignments of calf Rotavirus genome RNA species. VIROLOGY 117 435-443.

McCRAE MA, McCORQUODALE JG (1982b): The molecular biology of Rotaviruses IV. Molecular cloning of the bovine Rotavirus genome. JOURNAL OF VIROLOGY 44 1076-1079.

McCRAE MA, McCORQUODALE JG (1983a): The molecular biology of Rotaviruses V. Terminal structure of viral RNA species. VIROLOGY 126 204-212.

McCRAE MA, McCORQUODALE JG (1983b): Structural and functional studies on the Rotavirus genomic RNAs. In DS RNA VIRUSES. Edited by Compans RW and Bishop DHL. (Elsevier publishing co.) 83-93.

McCRAE MA (1987): The Reovirus family at the molecular level. Chapter 15 in The molecular basis of viral replication. Edited by Perez Bercoff R. Published by Plenum press (New York and London in cooperation with NATO scientific affairs division. p327-345.

McCRAE MA, McCORQUODALE JG (1987): Expression of a major bovine Rotavirus neutralisation antigen (VP7c) in Escherichia coli GENE 55 9-16.

McDONNELL MW, SIMON MN, STUDIER FW (1977): Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. JOURNAL OF MOLECULAR BIOLOGY. 110 119-125.

McLEAN BS, HOLMES IH (1980): Transfer of anti-rotaviral antibodies from mothers to their infants. JOURNAL OF CLINICAL MICROBIOLOGY 12 320-325.

McLEAN BS, HOLMES IH (1981): Effects of antibodies, trypsin and trypsin inhibitors on susceptibility of neonates to Rotavirus infection. JOURNAL OF CLINICAL MICROBIOLOGY 13 22-29.

McNULTY MS, ALLAN GM, McFERRAN JB (1967): Isolation of a cytopathic calf Rotavirus. RESEARCH IN VETERINARY SCIENCE 21 114-115.

McNULTY MS, ALLAN GM, CURRAN WL, McFERRAN JB (1976): Comparison of methods of diagnosis of Rotavirus infection in calves. VETERINARY RECORD 98 463-464.

McNULTY MS (1978): Reveiw article: Rotaviruses. JOURNAL OF GENERAL VIROLOGY 40 1-18.

McNULTY MS, ALLAN GM, TODD D, McFERRAN JB (1979): Isolation and cell culture of Rotaviruses from turkeys and chickens. ARCHIVES OF VIROLOGY 61 13-21.

McNULTY MS, ALLAN GM, TODD D, McFERRAN JB, McKILLOP ER, COLLINS DS, McCRACKEN RM (1980): Isolation of Rotaviruses from turkeys and chickens: demonstration of distinct serotypes and RNA electropherotypes. AVIAN PATHOLOGY 9 363-376.

McNULTY MS, ALLAN GM, TODD D, McFERRAN JB, McCRACKEN RM (1981): Isolation from chickens of a Rotavirus lacking the Rotavirus group antigen. JOURNAL OF GENERAL VIROLOGY 56 405-413.

McNULTY MS (1987): In discussion of: Novel Rotaviruses in animals and man. By BRIDGER JC in "Novel diarrhoea viruses". Ciba foundation symposium 128, published by Wiley and Sons 1987 p5-23.

MEBUS CA, UNDERDAHL NR, RHODES MB, TWEIHAUS MJ (1969): Calf diarrhoea (scours): Reproduced with a virus from a field outbreak. UNIVERSITY OF NEBRASKA AGRICULTURAL EXPERIMENTAL STATION RESEARCH BULLETIN 233.

MEBUS CA, KONO M, UNDERDAHL NR, TWEIHAUS MJ (1971a): Cell culture propagation of neonatal calf diarrhoea (scours) virus. CANADIAN VETERINARY JOURNAL 12 69-72.

MEBUS CA, STAIR EL, UNDERDAHL NR, TWEIHAUS MJ (1971b): Pathology of neonatal calf diarrhoea induced by a reo-like virus. VETERINARY PATHOLOGY 8 490-505.

MEBUS CA, NEWMAN LE (1977): Scanning electron, light and immunofluorescent microscopy of intestine of gnotobiotic calves infected with Reovirus-like agent. AMERICAN JOURNAL OF VETERINARY RESEARCH 38 553-558.

MEBUS CA, WYATT RG, KAPIKIAN AZ (1977): Intestinal lesions induced in gnotobiotic calves by the virus of human infantile gastroenteritis. VETINARY PATHOLOGY 14 273-282.

MELTON DA, KREIG PA, REBAGLIATI MR, MANIATIS T, ZINN K, GREEN MR (1984): Efficient in-vitro synthesis of biologically active RNA and RNA hybridisation probes from plasmids containing a bacteriophage SP6 promotor. NUCLEIC ACIDS RESEARCH 12 7035-7059.

MIDTHUN K, GREENBERG HB, HOSHINO Y, KAPIKIAN AZ, WYATT RG, CHANOCK RM (1985): Reassortant Rotaviruses as potential live Rotavirus vaccine candidates. JOURNAL OF VIROLOGY 53 949-954.

MIDTHUN K, HOSHINO Y, KAPIKIAN AZ, CHANOCK RM (1986): Single gene substitution rotavirus reassortants containing the major neutralising protein (VP7) of human Rotavirus serotype 4. JOURNAL OF CLINICAL MICROBIOLOGY 24 822-826.

MIURA KI, FUJII I, SAKAKI T, FUKE M, KANASE S (1968): Double stranded ribonucleic acid from cytoplasmic polyhedrosis virus of the silkworm. JOURNAL OF VIROLOGY 2 1211-1222.

MORGAN EM, ZWEERINK HJ (1974): Reovirus morphogenesis, Core-like particles in cells infected at 39 c with wild type reovirus and temperature sensitive mutants in groups B and G. VIROLOGY 59 556-565.

NAKATA S, ESTES MK, GRAHAM DY,WANG S, GARY GW, MELNICK JL (1987): Detection of antibody to Group B adult diarrhea virus in humans. JOURNAL OF CLINICAL MICROBIOLOGY 25 812-818.

NEWMAN JFE, BROWN F, BRIDGER JC, WOODE GN (1975): Characterisitcs of a Rotavirus. NATURE 258 631-633.

NICHOLS JL, BELLAMY AR, JOKLIK WK (1972): Identification of the nucleotide sequences of the oligonucleotides present in Reovirions. VIROLOGY 49 562-572.

NONOYAMA M, GRAHAM AF (1970): Appearance of defective virions in clones of Reovirus. JOURNAL OF VIROLOGY 6 693-694.

NONOYAMA M, MILLWARD S, GRAHAM AF (1974): Control of transcription of the Reovirus genome. NUCLEIC ACIDS RESEARCH 1 373-385.

OBIJESKI JF, PALMER EL, MARTIN MJ (1977): Biochemical characterisation of infantile gastroenteritis virus (IGV). JOURNAL OF GENERAL VIROLOGY 34 485-497.

OFFIT PA, CLARK HF, STROOP WG, TWIST EM, PLOTKIN SA (1983): The cultivation of human Rotavirus strain "Wa" to high titer in cell culture and characterisation of the viral structural polypeptides. JOURNAL OF VIROLOGICAL METHODS 7 29-40.

OFFIT PA, BLAVAT G (1986): Identification of the two Rotavirus genes determining neutralisation specificities. JOURNAL OF VIROLOGY 57 376-378.

OFFIT PA, BLAVAT G, GREENBERG HB, CLARK HF (1986a): Molecular basis of Rotavirus virulence: Role of Gene segment 4 JOURNAL OF VIROLOGY 57 46-49.

OFFIT PA, CLARK HF, BLAVAT G, GREENBERG HB (1986b): Reassortant Rotaviruses containing structural proteins VP3 and VP7 from different parents induce antibodies protective against each parental serotype. JOURNAL OF VIROLOGY 60 491-496.

OKADA Y, RICHARDSON MA, IKEGAMI N, NOMOTO A, FURUICHI Y (1984): Nucleotide sequence of human Rotavirus geneome segment 10, an RNA encoding a glycosylated virus protein. JOURNAL OF VIROLOGY 51 856-859.

PADGETT RA, HARDY SF, SHARP PA (1983): Splicing of adenovirus RNA in a cell-free transcription system. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES 80 5230-5234.

PALL CORPORATION (1985): Protocols for DNA and RNA transfer, DNA electotransfer and protein transfer to Biodyne A nylon membranes. Published by Pall Corporation and supplied with Biodyne A membrane.

PALMER EL, MARTIN ML, MURPHEY FA (1977): Morphology and stability of infantile gastroenteritis virus: Comparison with Reovirus and Bluetongue virus. JOURNAL OF GENERAL VIROLOGY 35 403-414.

PALMER EL, MARTIN ML, (1982): Further observations on the ultrastructure of human Rotavirus. JOURNAL OF GENERAL VIROLOGY 62 105-111.

PATTON JT (1986): Synthesis of Simian Rotavirus SA11 double-stranded RNA in a cell-free system. VIRUS RESEARCH 6 217-233.

PEDLEY S, BRIDGER JC, BROWN JF, McCRAE MA (1983): Molecular characterisation of Rotaviruses with distinct group antigens. JOURNAL OF GENERAL VIROLOGY 64 2093-2101.

PEDLEY S, HUNDLEY F, CHRYSTIE I, McCRAE MA, DESSELBERGER U (1984): The genomes of Rotavirus isolated from chronically infected immunodeficient children. JOURNAL OF GENERAL VIROLOGY 65 1141-1150.

PEDLEY S, McCRAE MA (1984): A rapid screening assay for detecting individual RNA species in field isolates of Rotaviruses. JOURNAL OF VIROLOGICAL METHODS 9 173-181.

PEDLEY S, BRIDGER JC, CHASEY D, McCRAE MA (1986): Definition of two new groups of atypical Rotaviruses. JOURNAL OF GENERAL VIROLOGY 67 131-137.

PEREIRA HG, AZEREDO RS, FIALHO AM, VIDAL MNP (1984): Genomic heterogeneity of Simian Rotavirus SA11. JOURNAL OF GENERAL VIROLOGY 68 653-660.

PEREZ-SCHAEL I, GONZALEZ M, DAOUD N, PEREZ M, SOTO G, GARCIA D, DAOUD G, KAPIKIAN AZ, FLORES J (1987): Reactogenicity and antigenicity of the rhesus rotavirus vaccine in Venezuelan children. JOURNAL OF INFECTIOUS DISEASES. 155 334-341.

PETRIE BL, GRAHAM DY, ESTES MK (1981): Identification of Rotavirus particle types. INTERVIROLOGY 16 20-28.

PETRIE BL, GRAHAM DY, HANSSEN H, ESTES MK (1982): Localisation of Rotavirus antigens in infected cells by ultrastructural immunocytochemistry. JOURNAL OF GENERAL VIROLOGY 63 457-467.

PETRIE BL, ESTES MK, GRAHMA DY (1983): Effects of tunicamycin on Rotavirus morphogenesis and infectivity. JOURNAL OF VIROLOGY 46 270-274.

PIWNICA-WORMS H, KEENE JD (1985): Replication of the Vesicular Stomatitis virus genome in permissive and non-permissive host cells. JOURNAL OF BIOLOGICAL CHEMISTRY. 260 10503-10511.

POCOCK DH (1987): Isolation and characterisation of two group A Rotaviruses with unusual genome profiles. JOURNAL OF GENERAL VIROLOGY 68 653-660.

POWELL KFH, HARVEY JD, BELLAMY AR (1984): Reovirus RNA transcriptase: Evidence for a conformational change during activation of the core particle. VIROLOGY 137 1-8.

RALPH SJ, HARVEY JD, BELLAMY AR (1980): Subunit structure of the Reovirus spike. JOURNAL OF VIROLOGY 36 894-896.

RODGER SM, SCHNAGL RD, HOLMES IH (1975): Biochemical and biophysical characteristics of diarrhea viruses of human and calf origin. JOURNAL OF VIROLOGY 16 1229-1235.

RODGER SM, SCHNAGL RD, HOLMES IH (1977): Further biochemical characterisation, including the detection of surface glycoproteins, of human, calf and simian Rotaviruses. JOURNAL OF VIROLOGY 24 91-98.

RODGER SM, BISHOP RF, BIRCH C, McLEAN, HOLMES IH (1981): Molecular epidemiology of human Rotaviruses in Melbourne Australia, from 1973-1979 as determined by electrophoresis of genome ribonucleic acid. JOURNAL OF CLINICAL MICROBIOLOGY 13 272-278.

RODRIGUES WJ, KIM HW, BRANDT CD, YOLKEN RH, ARROBIO JO, KAPIKIAN AZ, CHANOCK RM, PARROTT RM (1978): Sequential enteric illness associated with different Rotavirus serotypes. LANCET II 37.

RODRIGUES WJ, KIM HW, BRANDT CD, YOLKEN RH, RICHARD M, ARROBIO JA, SCHWARTZ RH, KAPIKIAN AZ, CHANOCK RM, PARROTT RM (1979): Common exposure outbreak of gastroenteritis due to type 2 Rotavirus with high secondary attack rate within families. JOURNAL OF INFECTIOUS DISEASE 140 353-357.

RUBIN DH, FIELDS BN (1980): Molecular basis of Reovirus virulence: Role of the M2 gene. JOURNAL OF EXPERIMENTAL MEDICINE 152 853-868.

RUECKERT RR (1985): Picornaviruses and their replication. Chapter 32 in "Virology" Edited by FIELDS BN. Published by Raven press, New York 705-738.

SABARA M, BABUIK LA, GILCHRIST J, MISRA V (1982a): Effect of tunicamycin on Rotavirus assembly and infectivity. JOURNAL OF VIROLOGY 43 1082-1090.

SABARA M, DEREGT D, BABUIK LA, MISRA V (1982b): Genetic heterogeneity within individual bovine Rotavirus isolates. JOURNAL OF VIROLOGY 44 813-822.

SACK DA, GILMAN RH, KAPIKIAN AZ, AZIZ KMS (1980): Serosepidemiology of Rotavirus infection in rural Bangladesh. JOURNAL OF CLINICAL MICROBIOLOGY 11 530-532.

SAIF LJ, THEIL KW, BOHL EH (1978): Morphogenesis of porcine Rotavirus in porcine kidney cell cultures and intestinal epithelial cells. JOURNAL OF GENERAL VIROLOGY 38 205-217.

SAIF LJ, BOHL EH, THEIL KW, CROSS RF, HOUSE JA (1980): Rotavirus-like, calicivirus-like, and 23nm virus-like particles associated with diarrhoea in young pigs. JOURNAL OF CLINICAL MICROBIOLOGY 12 105-111.

SAKUMA S, WATANABE Y (1972): Incorporation of in vitro synthesised Reovirus ds-RNA into virus corelike particles. JOURNAL OF VIROLOGY 10 943-950.

SANDINO AM, JASHES M, FAUNDEZ G, SPENCER E (1988): Role of the inner protein capsid in in-vitro human Rotavirus transcription. JOURNAL OF VIROLOGY 60 797-802.

SANTOSHAM M, DAUM RS, DILLMAN L, RODRIGUEZ JL, LUQUE S, RUSSELL R, KOURANG M, RYDER RW, BARTLETT AV, ROSENBERG A, BENENSON AS, SACK RB (1982): Oral rehydration therapy of infantile diarrhea. A controlled study of well nourished children hospitalised in the United States and Panama. NEW ENGLAND JOURNAL OF EXPERIMENTAL MEDICINE 306 1070-1076.

SCHNAGL RD, RODGER SM, HOLMES IH (1981): Variation in human Rotavirus electropherotypes occuring between Rotavirus gastroenteritis epidemics in Central Australia. INFECTION AND IMMUNITY 33 17-21.

SCHLESINGER MJ (1985): Replication of togaviruses. Chapter 43 in "Virology" Edited by FIELDS BN. Published by Raven press, New York 1021-1032.

SCHONBERG M, SILVERSTEIN SC, LEVIN DH, ACS G (1971): Asynchronous synthesis of the complementary strands of the Reovirus genome. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES 68 505-509.

SCHOUB BD, LECATAS G, PROZKY OW (1977): Antigenic relationship between human and simian Rotaviruses. JOURNAL OF MEDICAL MICROBIOLOGY 10 1-6.

SCHUERCH AR, MATSUHISA T, JOKLIK WK (1974): Temperature sensitive mutants of Reovirus VI. Mutant ts447 and ts556 particles that lack either one of two genome segments. INTERVIROLOGY 3 36-46.

SHAHRABADI MS, LEE PWK (1986): Bovine Rotavirus maturation is a calcium dependent process. VIROLOGY 152 298-307.

SHATKIN AJ, SIPE JD (1968a): Single-stranded adenine rich RNA from purified Reovirions. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 59 246-253.

SHATKIN AJ, SIPE JD (1968b): RNA polymerase activity in Reoviruses. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 61 1462-1469.

SHATKIN AJ, LaFIANDRA AJ (1972): Transcription by infectious subviral particles of Reovirus. JOURNAL OF VIROLOGY 10 698-706.

SHATKIN AJ (1974): Methylated messenger RNA synthesis in-vitro by purified Reovirions. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 71 3204-3207.

SHEPHERD RW, TRUSLOW S, WALKER-SMITH JA, BIRD R, CULTING W, DARNELL R, BARKER CM (1975): Infantile gastroenteritis: A clinical study of Reovirus-like agent infection. LANCET 2 1062-1063.

SHIRLEY JA, BEARDS GM, THOULESS ME, FLEWETT TH (1981): The influence of divalent cations on the stability of human Rotavirus. ARCHIVES OF VIROLOGY 67 1-10.

SILVERSTEIN SC, ASTELL C, LEVIN DH, SCHONBERG M, ACS G (1970): The mechanism of Reovirus uncoating and gene activation in vivo. VIROLOGY 47 797.

SILVERSTEIN SC, CHRISTMAN J, ACS G (1976): The Reovirus replicative cycle. ANNUAL REVIEWS OF BIOCHEMISTRY 45 375-408.

SILVERSTEIN SC, DALES S (1978): The penetration of Reovirus RNA and inactivation of its genetic function in L-strain fibroblasts. JOURNAL OF CELL BIOLOGY 36 197-230.

SKEHEL JJ, JOKLIK WK (1969): Studies on the in-vitro transcription of Reovirus RNA catalysed by Reovirus cores. VIROLOGY 39 822-831.

SKUP D, MILLWARD S (1980a): Reovirus induced modification of cap-dependent translation on infected cells. PROCEEDINGS OF THE NATIOAL ACADEMY OF SCIENCES USA 77 152-156.

SKUP D, MILLWARD S (1980b): mRNA capping enzymes are masked in Reovirus progeny sub-viral particles. JOURNAL OF VIROLOGY 34 490-496.

SMITH RE, ZWEERINK HJ, JOKLIK WK (1969): Polypeptide components of virions, top component and cores of Reovirus type 3. VIROLOGY 39 791-81.

SMITH ML, LAZDINS I, HOLMES IH (1980): Coding assignments of dsRNA segments of SA11 Rotavirus established by in vitro translation. JOURNAL OF VIROLOGY 33 976-982.

SNODGRASS DR, WELLS PW (1976): Rotavirus infection in lambs: Studies on passive protection. ARCHIVES OF VIROLOGY 52 201-206.

SNODGRASS DR, SMITH W, GRAY EW, HERRING JA (1976): A Rotavirus in lambs with diarrhoea. RESEARCH IN VETERINARY SCIENCE 20 113-114.

SNODGRASS DR, ANGUS KW, GRAY EW (1977): Rotavirus infection in lambs: Pathogenesis and pathology. ARCHIVES OF VIROLOGY 55 263-274.

SNODGRASS DR, HERRING JA (1977): The action of disinfectants on lamb Rotavirus. VETINARY RECORD 101 81.

SNODGRASS DR, WELLS PW (1978): Passive immunity in Rotaviral infections. JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION 173 565-568.

SNODGRASS DR, OJEH CK, CAMPBELL I, HERRING AJ (1984): Bovine Rotavirus serotypes and their significance for immunisation. JOURNAL OF CLINICAL MICROBIOLOGY 20 342-346.

SONZA S, HOLMES IH (1980): Coproantibody response to Rotavirus infection. MEDICAL JOURNAL OF AUSTRALIA 2 496-499.

SPANDIDOS DA, GRAHAM AF (1976): Non-permissive infection of L-cells by an avian Reovirus: Restricted transcription of the viral genome. JOURNAL OF VIROLOGY 19 977-984.

SPENCER E, ARIAS ML (1981): In vitro transcription catalysed by heat treated human Rotavirus. JOURNAL OF VIROLOGY 40 1-10.

STELLWAG EJ, DAHLBERG AE (1980): Electrophoretic transfer of DNA, RNA and protein onto Diazobenzyloxymethyl (DBM) paper. NUCLEIC ACIDS RESEARCH 8 299-317.

STRAUSS EG, STRAUSS JH (1982): Replication strategies of the single stranded RNA viruses of eukaryotes. In: CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY.Published by Springer Verlag. Berlin.

STRAUSS EG, RICE CM, STRAUSS JH (1983): Sequence coding for the alphavirus nonstructural proteins is interrupted by an opal termination codon. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 80 5271-5275.

STREET JE, CROXSON MC, CHADDERTON WF, BELLAMY AR (1982): Sequence diversity of human Rotavirus strains investigated by Northern Blot hybridisation analysis. JOURNAL OF VIROLOGY 43 369-378.

STUDIER FW, ROSENBERG AH (1981): Genetic and physical mapping of the late region of bacteriophage T7 DNA by use of cloned fragments of T7 DNA. JOURNAL OF MOLECULAR BIOLOGY 153 527-524.

SUZUKI H, KUTSUZAWA T, KONNO T, EBINA T, ISHIDA N (1981): Morphogenesis of human Rotavirus type 2 Wa strain in MA104 cells. ARCHIVES OF VIROLOGY 70 33-41.

SUZUKI H, SATO T, KONNO T, KITAUKA S, EBINA T, ISHIDA W (1984): Effect of tunicamycin on human Rotavirus morphogenesis and infectivity. ARCHIVES OF VIROLOGY 81 363-369.

SUZUKI H, KITAOKA S, KONNO T, SATO T, ISHIDA N (1985): Two modes of human Rotavirus entry into MA104 cells. ARCHIVES OF VIROLOGY 85 25-43.

SUZUKI H, KITAOKA S, SATO T, KONNO T, IWASAKI Y. NUMAZAKI Y, ISHIDA W (1986): Further investigation on the mode of entry of human Rotavirus into cells. ARCHIVES OF VIROLOGY 91 135-144.

TAM JS, KUM WWS, LAM B, YEUNG CY, NG MH (1986): Molecular epidemiology of human Rotavirus infection in children in Hong-Kong. JOURNAL OF CLINICAL MICROBIOLOGY 23 660-664.

TAN JA, SCHNAGL RD (1981): Inactivation of a Rotavirus by disinfectants. MEDICAL JOURNAL OF AUSTRALIA 1 19.

TERRETT LA, SAIF LJ (1987): Serial propagation of Porcine Group C Rotaviruses (pararotaviruses) in primary porcine kidney cell cultures. JOURNAL OF CLINICAL MICROBIOLOGY 25 1316-1319.

THEIL KW, BOHL EH, AGNES AG (1977): Cell culture propagation of porcine Rotavirus (Reovirus-like agent). AMERICAN JOURNAL OF VETERINARY RESEARCH 38 1765-1768.

THEIL KW, BOHL EH, CROSS RF, KOHLER EM, AGNES AG (1978a): Pathogenesis of porcine Rotaviral infection on experimentally inoculated gnotobiotic pigs. AMERICAN JOURNAL OF VETERINARY RESEARCH 39 213-220.

THEIL KW, BOHL EH, SAIF LJ (1978b): Techniques for Rotaviral propagation. JOURNAL OF THE AMERICAN VETERINARY MEDICAL ASSOCIATION 173 548-551.

THEIL KW, SAIF LJ, MOORHEAD PD, WHITMOYER RE (1985): Porcine Rotavirus-like agent (Group B Rotavirus): Characteristics and pathogenicity for gnotobiotic pigs. JOURNAL OF CLINICAL MICROBIOLOGY 21 340-345.

THOULESS ME, BRYDEN AS, FLEWETT TH (1977): Rotavirus neutralisation by human milk. BRITISH MEDICAL JOURNAL 2 1390-1394.

THOULESS ME, BRYDEN AS, FLEWETT TH (1978): Serotypes of human Rotavirus. LANCET I 39.

THOULESS ME, DIGIACOMO RF, NEWMAN DS (1986): Isolation of two lapine Rotaviruses: Characterisation of their subgroup, serotype and RNA electropherotypes. ARCHIVES OF VIROLOGY 89 161-170.

TODD D, McNULTY MS (1976): Characterisation of pig Rotavirus RNA. JOURNAL OF GENERAL VIROLOGY 33 147-150.

TODD D, McNULTY MS (1977): Biochemical studies of a Reovirus-like agent (Rotavirus) from lambs. JOURNAL OF VIROLOGY 21 1215-1218.

UHNOO I, SVENSSON L (1986): Clinical and epidemiological features of acute infantile gastroenteritis associated with human Rotavirus subgroups 1 and 2. JOURNAL OF CLINICAL MICROBIOLOGY 23 551-555.

VAN DYKE TA, RICKLES RJ, FLANAGAN JB (1982): Genome length copies of poliovirus RNA are synthesised in vitro by the poliovirus RNA dependent RNa polymerase. JOURNAL OF BIOLOGICAL CHEMISTRY 257 4610-4617.

VERLY E, COHEN J (1977): Demonstration of size variation of RNA segments between different isolates of calf Rotavirus. JOURNAL OF GENERAL VIROLOGY 35 583-586.

VERWOERD DW (1969): Purification and characterisation of bluetongue virus. VIROLOGY 38 203-212.

VESIKARI T, ISOLAURI E, DELEM A, d'HONDT E, ANDRE FE, ZISSIS G (1983): Immunogenicity and safety of live attenuated bovine Rotavirus vaccine strain RIT 4237 in adults and young children. LANCET II 907-911.

VESIKARI T, ISOLAURI E, d'HUNDT E, DELEM A, ANDRE FE, ZISSIS G (1984): Protection of infants against human Rotavirus diarrhea by the RIT 4237 live attenuated bovine Rotavirus vaccine. LANCET I 977-981.

VESIKARI T, ISOLAURI E, DELEM A, d'HONDT E, ANDRE FE, BEARDS GM, FLEWETT MD (1985): Clinical efficacy of the RIT 4237 live attenuated bovine Rotavirus vaccine in infants vaccinated before a Rotavirus epidemic. JOURNAL OF PEDIATRICS 107 189-194.

VESIKARI T, KAPIKIAN AZ, DELEM A, ZISSIS G (1986): A comparative trial of rhesus monkey (RRV-1) and bovine (RIT4237) oral rotavirus vaccines in young children. JOURNAL OF INFECTIOUS DISEASES 153 832-839.

VODOPIJA I, BAKLAIC Z, VLATKOVIC R, BOGAERTS, DELEM A, ANDRE FE (1986): Combined vaccination with live polio vaccine and the bovine Rotavirus RIT 4237 strain. VACCINE 4 233-236.

VON BONSDORFF EH, HOVI T, MAKELA P, HORI L, TEVALVOTO-AARN M (1976): Rotavirus associated with acute gastroenteritis in adults. LANCET II 423.

VONDERFECHT SL, HUBER AC, EIDEN J, MADER LC, YOLKEN RH (1984): Infectious diarrhoea of infant rats produced by a Rotavirus-like agent. JOURNAL OF VIROLOGY 52 94-98.

WALLACE RE, VASINGTON PJ, PETRICCIANI JC, HOPPS HE, LORENZ DE, KADANKA Z (1973): Development of a diploid cell line from fetal rhesus monkey lung for virus vaccine production. IN VITRO 8 323-332.

WATANABE Y, MILLWARD S, GRAHAM AF (1968): Regulation of transcription of the Reovirus genome. JOURNAL OF MOLECULAR BIOLOGY 36 107-123.

WEINBERG RJ, TIPTON G, KLISH WJ, BROWN MR (1984): Effect of breast feeding on morbidity in Rotavirus gastroenteritis. PEDIATRICS 74 250-253.

WEINER HL, DRAYNA D, AVERILL DR jn. FIELDS BN (1977): Molecular basis of Reovirus virulence: Role of the S1 gene. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA 74 5744-5748.

WEINER HL, FIELDS BN (1977): Neutralisation of Reovirus: The gene responsible for the neutralisation antigen. JOURNAL OF EXPERIMENTAL MEDICINE 146 1305-1310.

WEINER HL, RAMIG RF, MUSTOE TA, FIELDS BN (1978): Identification of the gene coding for the haemagglutinin of Reovirus. VIROLOGY 86 581-584.

WEINER HL, GREENE MI, FIELDS BN (1980): Delayed hypersensitivity in mice infected with Reovirus I. Identification of host and viral gene products responsible for the immune response. JOURNAL OF IMMUNOLOGY 125 278-282.

WENMAN WM, HINDE D, FELTHAM S, GURWITH M (1979): Rotavirus infection in adults: Results of a prospective family study. NEW ENGLAND JOURNAL OF MEDICINE 301 363.

WILLIAMS JG, MASON PJ (1985): Hybridisation in the analysis of RNA. Chapter 6 in: NUCLEIC ACID HYBRIDISATION, a PRACTICAL APPROACH. Edited by HAMES AND HIGGINS. IRL PRESS, OXFORD

WOODE GN, BRIDGER JC, HALL GA, DENNIS MJ (1974): The isolation of Reovirus-like agent associated with diarrhoea in colostrum deprived calves in Great Britain. RESEARCH IN VETERINARY SCIENCE 16 102-105.

WOODE GN, JONES J, BRIDGER JC, (1975): Levels of colostral antibodies against neonatal calf diarrhoea virus. VETERINARY RECORD 97 148.

WOODE GN, BRIDGER JC, JONES JM, FLEWETT TH, BRYDEN AS, DAVIES HA, WHITE GBB (1976): Morphological and antigenic relationships between viruses (Rotaviruses) from acute gastroenteritis of children, calves, piglets, mice and foals. INFECTION AND IMMUNITY 14 804-810.

WYATT RG, SLY GL, LONDON WT, PALMER AE, KALICA AR, VAN KIRK DH, CHANOCK RM, KAPIKIAN AZ (1976): Induction of diarrhea in colostrum deprived newborn rhesus monkeys with the human reovirus-like agent of infantile gastroenteritis. ARCHIVES OF VIROLOGY 50 17-27.

WYATT RG, YOLKEN RH, URRUTIA JJ, MATA L, GREENBERG HB, CHANOCK RM, KAPIKIAN AZ (1979a): Diarrhea associated with Rotavirus in rural Guatemala: A longitudinal study of 24 infants and young children. AMERICAN JOURNAL OF TROPICAL MEDICAL HYGIENE 28 325-328.

WYATT RG, MEBUS CA, YOLKEN RH, KALICA AR, JAMES HD Jr, KAPIKIAN AZ, CHANOCK RM, (1979b): Rotaviral immunity in gnotobiotic calves: heterologous resistance to human virus induced by bovine Rotavirus. SCIENCE 203 548-550.

WYATT RG, KAPIKIAN AZ, MEBUS CA (1979c): Induction of cross-reactive serum neutralising antibody to human Rotavirus in calves after in utero administration of bovine Rotavirus. JOURNAL OF MICROBIOLOGY 18 505-508.

WYATT RG, GREENBERG HB, JAMES WD, PITTMAN AL, KALICA AR, FLORES J, CHANOCK RM, KAPIKIAN AZ (1982): Definition of human Rotavirus serotypes by plaque reduction assay. INFECTION AND IMMUNITY 37 110-115.

YAMAKAWA M, FURUICHI Y, NAKASHIMA K, LaFIANDRA AJ (1981): Excess synthesis of viral mRNA 5'-terminal oligonucleotides by Reovirus transcriptase. JOURNAL OF BIOLOGICAL CHEMISTRY 256 6507-6514.

YAMAKAWA M, FURUICHI Y, SHATKIN AJ (1982): Reovirus transcriptase and capping enzymes are active in intact virions. VIROLOGY 118 157-168.

YOLKEN RH, MATA L, GARCIA B, URRUTIA JJ, WYATT RG, CHANOCK RM, KAPIKIAN AZ (1978): Secretory antibody directed against Rotavirus in human milk; measurement by means of Enzyme Linked Immunosorbent Assay. JOURNAL OF PEDIATRICS 93 916-921.

ZINN K, DIMAIO D, MANIATIS T (1983): Identification of two distinct regulatory regions adjacent to the human beta interferon gene. CELL 34 865-879

ZISSIS G, LAMBERT JP (1978): Different serotypes of human Rotavirus. LANCET I 38-39.

ZISSIS G, LAMBERT JB, MARBEHAN P (1983): Protection studies in colostrum deprived piglets of a bovine Rotavirus vaccine candidate using human Rotavirus strains for challenge. JOURNAL OF INFECTIOUS DISEASE. 148 1061-1068.

ZWEERINK HJ, JOKLIK WK (1970): Studies on the intracellular synthesis of Reovirus-specified proteins. VIROLOGY 41 501-518.

ZWEERINK HJ, McDOWELL MJ, JOKLIK WK (1971): Essential and non-essential non-capsid Reovirus proteins. VIROLOGY 45 716-723.

ZWEERINK HJ, (1974): Multiple forms of ss to ds RNA polymerase activity in Reovirus infected cells. NATURE (LONDON) 247 313-315.

ZWEERINK HJ, MORGAN EM, SKYLER JS (1976): Reovirus morphogenesis. Characterisation of subviral particles in infected cells. VIROLOGY 73 442-453.

ADDENDUM

The following paragraphs have been added to provide a fuller explanation of the points indicated in the text.

1. VP6. THE MAJOR ROTAVIRUS INNER CAPSID POLYPEPTIDE.

VP6 is the major Rotavirus inner capsid polypeptide and is responsible for subgroup specificity of viral isolates. Greenberg et al (1983) have isolated a series of 10 monoclonal antibodies directed at VP6 by immunising mice with either Wa or RRV Rotavirus strains. Using this set of monoclonal antibodies it has been shown that a porcine and an equine rotavirus strain exhibited the subgroup 2 specificity, which had previously only been detected in human Rotaviruses (Kapikian et al 1981) and that the EDIM virus and Turkey Rotaviruses are antigenically distinct from other mammalian Rotavirus strains (Greenberg et al 1983). The data presented by Greenberg et al (1983) supports the proposition that there are at least two distinct antigenic domains on the 42K protein. The first domain is shared among all mammalian Rotaviruses, while the second, and the one detected by the monoclonal antibodies described above can have at least two specificities (Subgroups 1 and 2). It is this antigenic domain of VP6 that the primary immune response is mounted when gnotobiotic calves are infected with Rotavirus, while hyperimmune sera contains antibodies to both domains. The

monoclonal antibodies isolated in this study should provide a means of studying viral assembly and structure (Greenberg et al 1983).

The structure of the VP6 subunits on the inner capsid shell has been studied by two groups (Gorziglia et al 1985 and Sabara et al 1987) and it has been illustrated that VP6 can exist in a trimeric form, the monomers being held together by non-covalent interactions (Gorziglia et al 1985). These trimeric units may complex further by disulphide bonding into larger subunits which may represent the hexameric structures observed by electron microscopy (see Figure 1), and their formation appears to be independent of RNA-protein interactions (Sabara et al 1987). The proposition the there are two antigentic domains of VP6 described above (Greenberg et al 1982) is supported by Sabara et al (1987) who have isolated antibodies which react to the oligomeric forms of VP6 of viruses belonging to the two different subgroups. This group have also found that these oligomeric forms of VP6 are exposed on the surface of the virus following assembly and elicit the formation of antibodies with a low level of neutralising activity (Sabara et al 1987).

2. GENE REASSORTMENT.

Recent evidence refutes the statement given on Page 18 of this manuscript that Rotavirus gene-reassortment is random. Graham et al (1987) have demonstrated that when reassortants between human Rotaviruses (carrying rearranged genomes) and bovine Rotavirus are made by mixed infection of tissue culture

cells, the emergence of reassortants was not random and the host cell used to isolate the reassortants (MA104 or BSC-1) had a selective effect on the recombinational mixture (Graham et al 1987). Ward et al (1988) have studied the selection of reassortants following coinfection of cultured cells with pairs of Subgroup 2 human Rotaviruses. They found that among the reassortants more segments were selected from the virus which grew to a higher titer, and that certain segments were selected independently of the relative growth properties or multiplicities of infection of the coinfecting viruses, while selection of others was dependant on one or both of these factors. These authors also found that selection of specific reassortants following co-infection was apparently due to differences in the infectivities of the progeny viruses and not in their assembly, and implies that the infectivities were a function of the parental origin of specific genome segments (Ward et al 1988).

EVIDIENCE FOR GENE REASSORTMENT IN-VIVO.

In 1986 Gombold and Ramig reported the isolation of reassortant Rotaviruses from the intestinal homogenates of mice inoculated with a mixture of wild-type SA11 and RRV Rotaviruses. From kinetic studies they concluded that reassortment was an early event in the infectious cycle and that although most segments reassort at random, segments 3 and 5 were non-randomly segregated from the SA11 parent (Gombold and Ramig 1986).

ADDITIONAL REFERENCES.

DESSELBERGER U, HUNG T, FOLLETT EAC (1986): Genome analysis of human Rotaviruses by oligonucleotide mapping of isolated RNA segments. VIRUS RESEARCH 4 357-368.

GARBARG-CHENON A, BRICOUT F, NICOLAS JC (1986): Serological characterisation of human reassortant rotaviruses. JOURNAL OF VIROLOGY 59 510-513.

GOMBOLD JL, RAMIG RF (1986): Analysis of reassortment of genome segments in mice mixedly infected with Rotavirus SA11 and RRV. JOURNAL OF VIROLOGY 57 110-116.

GORZIGLIA C, LARREA C, LIPRANDI F, ESPARZA J (1985): Biochemical evidence for the oligomeric (possibly trimeric) structure of the major inner capsid polypeptide (45K) of rotaviruses. JOURNAL OF GENERAL VIROLOGY 66 1899-1900.

GRAHAM A, KUDESIA G, ALLEN AM, DESSELBERGER U (1987): Reassortment of human Rotaviruses possessing genome rearrangements with bovine Rotavirus: Evidence for host cell selection. JOURNAL OF GENERAL VIROLOGY 68 115-122.

IMAI M, AKATARI K, IKEGAMI N, FURUICHI Y (1983): Capped and conserved terminal structures in human Rotavirus genome double-stranded RNA segments. JOURNAL OF VIROLOGY 47 125-136.

KAPIKIAN AZ, CLINE WL, GREENBERG HB, WYATT RG, KALICA AR, BANKS CC, JAMES HD Jnr, FLORES J, CHANOCK RM (1981): Antigenic characterisation of human and animal rotaviruses by immune adherence haemagglutination assay (IAHA); Evidence for distinctness of IAHA and neutralisation antigens. INFECTION AND IMMUNITY 33 415-425.

KUTSUZAWA T, KONNO Y, SUZUKI H, KAPIKIAN AZ, EBINA T, ISHIDA N (1982): Isolation of human Rotavirus subgroups 1 and 2 in cell culture. JOURNAL OF CLINICAL VIROLOGY 18 727-730.

LIU M, OFFIT PA, ESTES MK (1988): Identification of the simian rotavirus SA11 genome segment 3 protein. VIROLOGY 163 26-32.

OKAYAMA H, BERG P (1982): High efficiency cloning of full length cDNA. MOLECULAR AND CELLULAR BIOLOGY 2 161-170.

RIXON F, TAYLOR P, DESSELBERGER U (1984): Rotavirus RNA segments sized by electron microscopy. JOURNAL OF GENERAL VIROLOGY 65 233-238.

SABARA M, READY KFM, FRENCHICK PJ, BABIUK LA (1987): Biochemical evidence for the oligomeric arrangement of bovine rotavirus nucleocapsid protein and its possible significance in the immunogenicity of the protein. JOURNAL OF GENERAL VIROLOGY 68 123-133.

SATO K, INABA Y,SHINOZAKI T, FUJII R, MATUMOTO M (1981): Isolation of human Rotavirus in cell cultures. A brief report. ARCHIVES OF VIROLOGY. 69 155-160.

SNODGRASS DR, HERRING AJ, CAMPBELL I, INGLIS JM, HARGREAVES FD (1984): Comparison of atypical Rotaviruses from calves, piglets, lambs and man. JOURNAL OF GENERAL VIROLOGY 65 909-914.

WARD RL, KNOWLTON DR, HURST P-F L (1988): Reassortant formation and selection following coinfection of cultured cells with subgroup 2 human rotaviruses. JOURNAL OF GENERAL VIROLOGY 69 149-162.

TITLE KINETICS OF RNA SYNTHESIS IN ROTAVIRUS INFECTED CELLS.

AUTHOR Moira A. Johnson.

INSTITUTION and DATE University of Warwick. 1988